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(71) Applicant: OPHIDIAN PHARMACEUTICALS [US/US]; 5445 East Cheryl Parkway, Madison, WI 53711 (US).	
(72) Inventors: CARROLL, Sean, B.; 3066 Streb Way, Cottage Grove, WI 53527 (US). STAFFORD, Douglas, C.; 21 Laramie Court, Madison, WI 53719 (US). PADHYE, Nisha, V.; 5743 Timber View Court, Fitchburg, WI 53711 (US).	
(74) Agents: CARROLL, Peter, G. et al.; Medlen & Carroll, Suite 2200, 220 Montgomery Street, San Francisco, CA 94104 (US).	

(54) Title: **TREATMENT FOR VEROTOXIN-PRODUCING ESCHERICHIA COLI**

(57) Abstract

The present invention includes methods for generating neutralizing antitoxin directed against verotoxins. In particular, the antitoxin directed against these toxins is produced in avian species using soluble recombinant verotoxin proteins. This avian antitoxin is designed so as to be administrable in therapeutic amounts and may be in any form (i.e., as a solid or in aqueous solution). These antitoxins are useful in the treatment of humans and other animals intoxicated with at least one bacterial toxin, as well as for diagnostic assays to detect the presence of toxin in a sample.

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TREATMENT FOR VEROTOXIN-PRODUCING *ESCHERICHIA COLI*

FIELD OF THE INVENTION

The present invention relates to antitoxin therapy for humans and other animals, and diagnostic assays to detect toxins. Antitoxins which neutralize the pathologic effects of *Escherichia coli* toxins, such as verotoxin are provided.

BACKGROUND OF THE INVENTION

A. *Escherichia coli* as a Pathogenic Organism

Escherichia coli is the organism most commonly isolated in clinical microbiology laboratories, as it is usually present as normal flora in the intestines of humans and other animals. However, it is an important cause of intestinal, as well as extraintestinal infections. For example, in a 1984 survey of nosocomial infections in the United States, *E. coli* was associated with 30.7% of the urinary tract infections, 11.5% of the surgical wound infections, 15 6.4% of the lower respiratory tract infections, 10.5% of the primary bacteremia cases, 7.0% of the cutaneous infections, and 7.4% of the other infections (J.J. Farmer and M.T. Kelly, "Enterobacteriaceae." in Manual of Clinical Microbiology, Balows *et al.*(eds). American Society for Microbiology, [1991], p. 365). Surveillance reports from England, Wales and Ireland for 1986 indicate that *E. coli* was responsible for 5,473 cases of bacteremia (including blood, bone marrow, spleen and heart specimens); of these, 568 were fatal. For spinal fluid specimens, there were 58 cases, with 10 fatalities (J.J. Farmer and M.T. Kelly, "Enterobacteriaceae." in Manual of Clinical Microbiology, Balows *et al.*(eds). American Society for Microbiology, [1991], p. 366). There are no similar data for United States, as these are not reportable diseases in this country.

Studies in various countries have identified certain serotypes (based on both the O and H antigens) that are associated with the four major groups of *E. coli* recognized as enteric pathogens. Table 1 lists common serotypes included within these groups. The first group includes the classical enteropathogenic serotypes ("EPEC"); the next group includes those that produce heat-labile or heat-stable enterotoxins ("ETEC"); the third group includes the enteroinvasive strains ("EIEC") that mimic *Shigella* strains in their ability to invade and multiply within intestinal epithelial cells; and the fourth group includes strains and serotypes that cause hemorrhagic colitis or produce Shiga-like toxins (or verotoxins) ("VTEC" or "EHEC" [enterohemorrhagic *E. coli*]).

Table 1.
Pathogenic *E. coli* Serotypes

Group	Associated Serotypes
5 Enterotoxigenic (ETEC)	O6:H16; O8:NM; O8:H9; O11:H27; O15:H11; O20:NM; O25:NM; O25:H42; O27:H7; O27:H20; O63:H12; O78:H11; O78:H12; O85:H7; O114:H21; O115:H21; O126:H9; O128ac:H7; O128ac:H12; O128ac:H21; O148:H28; O149:H4; O159:H4; O159:H20; O166:H27; and O167:H5
Enteropathogenic (EPEC)	O26:NM; O26:H11; O55:NM; O55:H6; O86:NM; O86:H2; O86:H34; O111ab:NM; O111ab:H2; O111ab:H12; O111ab:H21; O114:H2; O119:H6; O125ac:H21; O127:NM; O127:H6; O127:H9; O127:H21; O128ab:H2; O142:H6; and O158:H23
Enteroinvasive (EIEC)	O28ac:NM; O29:NM; O112ac:NM; O115:NM; O124:NM; O124:H7; O124:H30; O135:NM; O136:NM; O143:NM; O144:NM; O152:NM; O164:NM; and O167:NM
10 Verotoxin-Producing (VTEC))	O1:NM; O2:H5; O2:H7; O4:NM; O4:H10; O5:NM; O5:H16; O6:H1; O18:NM; O18:H7; O25:NM; O26:NM; O26:H11; O26:H32; O38:H21; O39:H4; O45:H2; O50:H7; O55:H7; O55:H10; O82:H8; O84:H2; O91:NM; O91:H21; O103:H2; O111:NM; O111:H8; O111:H30; O111:H34; O113:H7; O113:H21; O114:H48; O115:H10; O117:H4; O118:H12; O118:H30; O121:NM; O121:H19; O125:NM; O125:H8; O126:NM; O126:H8; O128:NM; O128:H2; O128:H8; O128:H12; O128:H25; O145:NM; O125:H25; O146:H21; O153:H25; O157:NM; O157:H7; O163:H19; O165:NM; O165:19; and O165:H25

B. Verotoxin Producing Strains of *E. coli*

15 Although all of these disease-associated serotypes cause potentially life-threatening disease, *E. coli* O157:H7 and other verotoxin-producing strains have recently gained widespread public attention in the United States due to their recently recognized association with two serious extraintestinal diseases, hemolytic uremic syndrome ("HUS") and thrombotic thrombocytopenic purpura ("TTP"). Worldwide, *E. coli* O157:H7 and other verotoxin-producing *E. coli* (VTEC) are an increasingly important human health problem. First identified as a cause of human illness in early 1982 following two outbreaks of food-related hemorrhagic colitis in Oregon and Michigan (M.A. Karmali, "Infection by Verocytotoxin-Producing *Escherichia coli*," Clin. Microbiol. Rev., 2:15-38 [1989]; and L. W. Riley, *et al.* "Hemorrhagic colitis associated with a rare *Escherichia coli* serotype," New Eng. J. Med.,

308: 681-685 [1983]). the reported incidence of VTEC-associated disease has risen steadily, with outbreaks occurring in the U.S., Canada, and Europe.

With increased surveillance, *E. coli* O157:H7 has been recognized in other areas of the world including Mexico, China, Argentina, Belgium, and Thailand (N. V. Padhye and M. P. Doyle, "Escherichia coli O157:H7: Epidemiology, pathogenesis and methods for detection in food," *J. Food. Prot.*, 55: 555-565 [1992]; and P. M. Griffin and R. V. Tauxe, "The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome," *Epidemiol. Rev.*, 13: 60 [1991]).

The disease attracted national attention in the U.S. after a major outbreak in the Pacific Northwest that was associated with consumption of undercooked *E. coli* O157:H7-contaminated hamburgers. Over 700 hundred people fell ill (more than 170 were hospitalized) and four young children died (P. Recer, "Experts call for irradiation of meat to protect against food-borne bacteria," Associated Press, 7/12/94 [1994]). Several outbreaks since then have underscored the potential severity and multiple mechanisms for transmission of VTEC-associated diseases (M. Bielaszewska *et al.*, "Verotoxigenic (enterohaemorrhagic) *Escherichia coli* in infants and toddlers in Czechoslovakia," *Infection* 18: 352-356 [1990]; A. Caprioli *et al.*, "Hemolytic-uremic syndrome and Vero cytotoxin-producing *Escherichia coli* infection in Italy," *J. Infect. Dis.*, 166: 184-158 [1992]; A. Caprioli, *et al.*, "Community-wide Outbreak of Hemolytic-Uremic Syndrome Associated with Non-O157 Verocytotoxin-Producing *Escherichia coli*," *J. Infect. Dis.*, 169: 208-211 [1994]; N. Cimolai, "Low frequency of high level Shiga-like toxin production in enteropathogenic *Escherichia coli* serogroups," *Eur. J. Pediatr.*, 151: 147 [1992]; and R. Voelker, "Panel calls *E. coli* screening inadequate," *Escherichia coli* O157:H7--Panel sponsored by the American Gastroenterological Association Foundation in July 1994, *Medical News & Perspectives*, *J. Amer. Med. Assoc.*, 272: 501 [1994]).

While O157:H7 is currently the predominant *E. coli* serotype associated with illness in North America, other serotypes (as shown in Table 1, and in particular O26:H11, O113:H21, O91:H21 and O111:NM) also produce verotoxins which appear to be important in the pathogenesis of gastrointestinal manifestations and the hemolytic uremic syndrome (P. M. Griffin and R. V. Tauxe, "The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome," *Epidemiol. Rev.*, 13: 60 [1990]; M. M. Levine, *et al.*, "Antibodies to Shiga holotoxin and to two synthetic peptides of the B subunit in sera of patients with *Shigella dysenteriae* 1

dysentery." *J. Clin. Microbiol.*, 30: 1636-1641 [1992]; and C. R. Dorn, *et al.*, "Properties of Vero cytotoxin producing *Escherichia coli* of human and animal origin belonging to serotypes other than O157:H7," *Epidemiol. Infect.*, 103: 83-95 [1989]). Since organisms with these serotypes have been shown to cause illness in humans they may assume greater public health 5 importance over time (P. M. Griffin and R. V. Tauxe, "The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome," *Epidemiol. Rev.*, 13: 60 [1990]).

Clinicians usually observe cases of hemolytic uremic syndrome ("HUS") clustered in a geographic region. However, small outbreaks are likely to be missed because many 10 laboratories do not routinely screen stool specimens for *E. coli* O157:H7. Many cases related to non-commercial food preparation also probably go unrecognized. Nonetheless, *E. coli* O157:H7 is responsible for a large number of cases, as more than 20,000 cases of *E. coli* O157:H7 infection are reported annually in the U.S., with 400-500 deaths from HUS. 15 However, these estimates were compiled when only 11 states mandated reporting of *E. coli* O157:H7. Twenty-nine states have recently made *E. coli* O157:H7 infection a reportable disease (R. Voelker, "Panel calls *E. coli* screening inadequate: *Escherichia coli* O157:H7; panel sponsored by the American Gastroenterological Association Foundation in July 1994, Medical News & Perspectives," *J. Amer. Med. Assoc.*, 272: 501 [1994]). Indeed, the Centers for Disease Control recently added *E. coli* O157:H7 to their list of reportable diseases 20 ("Public Health Threats," *Science* 267:1427 [1995]).

C. Nature of Verotoxin-Induced Disease

Risk factors for HUS progression following infection with *E. coli* O157:H7 include age (very young or elderly), bloody diarrhea, leukocytosis, fever, large amounts of ingested 25 pathogen, previous gastrectomy, and the use of antimicrobial agents (in particular, trimethoprim-sulfamethoxazole)(A. A. Harris *et al.*, "Results of a screening method used in a 12 month stool survey for *Escherichia coli* O157:H7," *J. Infect. Dis.*, 152: 775-777 [1985]; and M. A. Karmali, "Infection by Verocytotoxin-producing *Escherichia coli*," *Clin. Microbiol. Rev.*, 2: 15-38 [1989]).

As indicated above, *E. coli* O157:H7 is associated with significant morbidity and 30 mortality. The spectrum of illness associated with *E. coli* O157:H7 infection includes asymptomatic infection, mild uncomplicated diarrhea, hemorrhagic colitis, HUS, and TTP". Hemorrhagic colitis (or "ischemic colitis") is a distinct clinical syndrome characterized by

sudden onset of abdominal cramps—likened to the pain associated with labor or appendicitis—followed within 24 hours by watery diarrhea. One to two days later, the diarrhea turns grossly bloody in approximately 90% of patients and has been described as "all blood and no stool" (C. H. Pai *et al.*, "Sporadic cases of hemorrhagic colitis associated with 5 *Escherichia coli* O157:H7," *Ann. Intern. Med.*, 101: 738-742 [1984]; and R. S. Remis *et al.*, "Sporadic cases of hemorrhagic colitis associated with *Escherichia coli* O157:H7," *Ann. Intern. Med.*, 101: 738-742 [1984]). Vomiting may occur, but there is little or no fever. The 10 time from ingestion to first loose stool ranges from 3-9 days (with a mean of 4 days) L. W. Riley *et al.*, "Hemorrhagic colitis associated with a rare *Escherichia coli* serotype," *New Eng. J. Med.*, 308: 681-685 [1983]; and D. Pudden *et al.*, "Hemorrhagic colitis in a nursing home," *Ontario Can. Dis. Weekly Rpt.*, 11: 169-170 [1985]), and the duration of illness ranges 15 generally from 2-9 days (with a mean of 4 days).

HUS is a life-threatening blood disorder that appears within 3-7 days following onset 20 of diarrhea in 10-15% of patients. Those younger than 10 years and the elderly are at particular risk. Symptoms include renal glomerular damage, hemolytic anemia (rupturing of erythrocytes as they pass through damaged renal glomeruli), thrombocytopenia and acute 25 kidney failure. Approximately 15% of patients with HUS die or suffer chronic renal failure. Indeed, HUS is a leading cause of renal failure in childhood (reviewed by M.A. Karmali, "Infection by Verocytotoxin-producing *Escherichia coli*," *Clin. Microbiol. Rev.*, 2: 15-38 30 [1989]). Currently, blood transfusion and dialysis are the only therapies for HUS.

TTP shares similar histopathologic findings with HUS, but usually results in 25 multiorgan microvascular thrombosis. Neurological signs and fever are more prominent in TTP, compared with HUS. Generally occurring in adults, TTP is characterized by microangiopathic hemolytic anemia, profound thrombocytopenia, fluctuating neurologic signs, 30 fever and mild azotemia (H. C. Kwaan, "Clinicopathological features of thrombotic thrombocytopenic purpura," *Semin. Hematol.*, 24: 71-81 [1987]; and S. J. Machin, "Clinical annotation: Thrombotic thrombocytopenic purpura," *Br. J. Hematol.*, 56: 191-197 [1984]). Patients often die from microthrombi in the brain. In one review of 271 cases, a rapidly progressive course was noted, with 75% of patients dying within 90 days (E.L. Amorosi and 35 J.E. Ultmann, "Thrombotic thrombocytopenic purpura: Report of 16 cases and review of the literature," *Med.*, 45:139-159 (1966)).

Other diseases associated with *E. coli* O157:H7 infection include hemorrhagic cystitis and balantitis (W. R. Grandson *et al.*, "Hemorrhagic cystitis and balantitis associated with

verotoxin-producing *Escherichia coli* O157:H7." *Lancet* ii: 150 [1985]). convulsions, sepsis with other organisms and anemia (P. C. Rowe *et al.*, "Hemolytic anemia after childhood *Escherichia coli* O157:H7 infection: Are females at increased risk?" *Epidemiol. Infect.*, 106: 523-530 [1991]).

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D. Mechanism of Pathogenesis

Verotoxins are strongly linked to *E. coli* O157:H7 pathogenesis. All clinical isolates of *E. coli* O157:H7 have been shown to produce one or both verotoxins (VT1 and VT2) (C. A. Bopp *et al.*, "Unusual Verotoxin-producing *Escherichia coli* associated with hemorrhagic colitis." *J. Clin. Microbiol.*, 25: 1486-1489 [1987]). Both of these toxins are cytotoxic to Vero (African green monkey kidney) and HeLa cells, and cause paralysis and death in mice (A. D. O'Brien *et al.*, "Purification of *Shigella dysenteriae* 1 (Shiga) like toxin from *Escherichia coli* O157:H7 strain associated with hemorrhagic colitis." *Lancet* ii: 573 [1983]). These toxins are sometimes referred to in the literature as Shiga-like toxins I and II (SLT-I and SLT-II, respectively), due to their similarities with the toxins produced by *Shigella*. Indeed, much of our understanding of *E. coli* VTs is based on information accumulated on Shiga toxins. Shiga toxin, first described in 1903, has been recognized as one of the most potent bacterial toxins for eukaryotic cells (reviewed by M.A. Karmali, "Infection by Verocytotoxin-producing *Escherichia coli*," *Clin. Microbiol. Rev.*, 2: 15-38 [1989]).

20 Hereinafter, the VT convention will be used; thus, VT1 and VT2 correspond to SLT-I and SLT-II, respectively.

While the pathogenic mechanism of *E. coli* O157:H7 infection is incompletely understood, it is believed that ingested organisms adhere to and colonize the intestinal mucosa, where toxins are released which cause endothelial cell damage and bloody diarrhea. It is also postulated that hemorrhagic colitis progresses to HUS when verotoxins enter the bloodstream, damaging the endothelial cells of the microvasculature and triggering a cascade of events resulting in thrombus deposition in small vessels. These microthrombi occlude the microcapillaries of the kidneys (particularly in the glomeruli) and other organs, resulting in their failure (J. J. Byrnes and J. L. Moake, "TTP and HUS syndrome: Evolving concepts of pathogenesis and therapy," *Clin. Hematol.*, 15: 413-442 [1986]; and T. G. Cleary, "Cytotoxin-producing *Escherichia coli* and the hemolytic uremic syndrome," *Pediatr. Clin. North Am.*, 35: 485-501 [1988]). Verotoxins entering the bloodstream may also result in direct kidney cytotoxicity.

VT1 is immunologically and structurally indistinguishable from Shiga toxin produced by *Shigella dysenteriae* (A. D. O'Brien *et al.*, "Purification of *Shigella dysenteriae* 1 (Shiga) like toxin from *Escherichia coli* O157:H7 strain associated with hemorrhagic colitis." *Lancet* ii: 573 [1983]). VT1 and VT2 holotoxins each consist of one A and five B subunits (A. 5 Donohue-Rolfe *et al.*, "Purification of Shiga toxin and Shiga-like toxins I and II by receptor analog affinity chromatography with immobilized P1 glycoprotein and production of cross reactive monoclonal antibodies." *Infect. Immun.* 57: 3888-3893 [1989]; and A. Donohue-Rolfe *et al.*, "Simplified high yield purification of *Shigella* toxin and characterization of 10 subunit composition and function by the use of subunit-specific monoclonal and polyclonal antibodies." *J. Exp. Med.* 160: 1767-1781 [1984]). The toxic A subunit is enzymatically active, while the B subunit binds the holotoxin to the receptor on the target eukaryotic cell.

15 Crystal structure analysis of Shiga holotoxin and VT1 B subunit pentamers have shown that the holotoxin assembles with the C-terminal end of the A subunit associating with, and inserting within, a pentamer of B chains (P. E. Stein *et al.*, "Crystal structure of the cell-binding B oligomer of verotoxin-1 from *E. coli*." *Nature* 355: 748-750 [1992]; and M.E. Fraser *et al.*, "Crystal structure of the holotoxin from *Shigella dysenteriae* at 2.5 Å resolution." *Struct. Biol.* 1:59-64 [1994]). This conformation is consistent with the observation that a C-terminally truncated A1 subunit of VT1 is toxic (in a ribosomal inhibition assay), but cannot associate with B subunit pentamers (P. R. Austin *et al.*, "Evidence that the A₁ fragment of 20 Shiga-like toxin type I is required for holotoxin integrity." *Infect. Immun.* 62: 1768 [1994]).

25 **The Verotoxin A Subunit.** Examination of the crystal structure of Shiga holotoxin indicates that the N-terminus of its A subunit is both surface-exposed and functionally important. Removal of amino acid interval 3-18 of the A subunit completely abolished toxicity (L. P. Perera *et al.*, "Mapping the minimal contiguous gene segment that encodes functionally active Shiga-like toxin II." *Infect. Immun.* 59: 829-835 [1991]) while removal of interval 25-44 retained toxicity but abolished its association with B subunit pentamers (J. E. Haddad *et al.*, "Minimum domain of the Shiga toxin A subunit required for enzymatic activity." *J. Bacteriol.* 175: 4970-4978 [1993]). Deletion of the first 13 residues of the homologous ricin A subunit also abolished toxicity, while deletion of the first 9 residues did 30 not (M. J. May, *et al.*, "Ribosome inactivation by ricin A chain: A sensitive method to assess the activity of wild-type and mutant polypeptides." *EMBO J.* 8: 301-308 [1989]).

The Verotoxin B Subunit. Studies of Shiga toxin B subunit suggest that neutralizing epitopes may also be present at both the N- and C-terminal regions of VT1 and VT2 B

subunits. Polyclonal antibodies raised against peptides from these regions (residues 5-18, 13-26, 7-26, 54-67 and 57-67) show partial neutralization of Shiga toxin (I. Harari and R. Arnon, "Carboxy-terminal peptides from the B subunit of Shiga toxin induce a local and parenteral protective effect." *Mol. Immunol.*, 27: 613-621 [1990]; and I. Harari *et al.*, 5 "Synthetic peptides of Shiga toxin B subunit induce antibodies which neutralize its biological activity." *Infect. Immun.*, 56: 1618-1624 [1988]). Deletion of the last five amino acids of Shiga toxin B (M. P. Jackson *et al.*, "Functional Analysis of the Shiga toxin and Shiga-like toxin Type II variant binding subunits by using site-directed mutagenesis." *J. Bacteriol.*, 172: 653-658 [1990]), or four amino acids of VT2 B (L. P. Perera *et al.*, "Mapping the minimal 10 contiguous gene segment that encodes functionally active Shiga-like toxin II." *Infect. Immun.*, 59: 829-835 [1991]), eliminate toxin activity, while deletion of the last two amino acids of VT2 B subunit reduced cytotoxicity. In contrast, the addition of an 18 or 21 amino acid extension to the native C-terminus of the VT2 B subunit was presumably conformationally correct, as these proteins assembled cytotoxic holotoxin.

15 Various approaches to express recombinant verotoxins have included individual or coordinate expression of A and B subunits from high-copy number plasmids and expression with fusion partners (J. E. Haddad *et al.*, "Minimum domain of the Shiga toxin A subunit required for enzymatic activity." *J. Bacteriol.*, 175: 4970-4978; J. E. Haddad, and M. P. Jackson, "Identification of the Shiga toxin A-subunit residues required for holotoxin 20 assembly." *J. Bacteriol.*, 175: 7652-7657 [1993]; M. P. Jackson *et al.*, "Mutational analysis of the Shiga toxin and Shiga-like toxin II enzymatic subunits." *J. Bacteriol.*, 172: 3346-3350 [1990]; C. J. Hovde *et al.*, "Evidence that glutamic acid 167 is an active-site residue of Shiga-like toxin I." *Proc. Natl. Acad. Sci.*, 85: 2568-2572 [1988]; R. L. Deresiewicz *et al.*, "The role of tyrosine-114 in the enzymatic activity of the Shiga-like toxin I A-chain." *Mol. Gen. Genet.*, 241: 467-473 [1993]; T. M. Zollman *et al.*, "Purification of Recombinant Shiga-like Toxin Type I A, Fragment from *Escherichia coli*." *Protein Express.Purific.*, 5: 291-295 [1994]; K. Ramotar, *et al.*, "Characterization of Shiga-like toxin I B subunit purified from overproducing clones of the SLT-I B cistron." *Biochem J.*, 272: 805-811 [1990]; S. B. Calderwood *et al.*, "A system for production and rapid purification of large amounts of the 25 Shiga toxin/Shiga-like toxin I B subunit." *Infect. Immun.*, 58: 2977-2982 [1990]; D. W. K. Acheson, *et al.*, "Comparison of Shiga-like toxin I B-subunit expression and localization in *Escherichia coli* and *Vibrio cholerae* by using *trc* or Iron-regulated promoter systems." *Infect. Immun.*, 61: 1098-1104 [1993]; M. P. Jackson *et al.*, "Nucleotide sequence analysis and 30

comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from *Escherichia coli* 933," FEMS Microbiol. Lett., 44: 109-114 [1987]; J. W. Newland *et al.*, "Cloning of genes for production of *Escherichia coli* Shiga-like toxin type II," Infect. Immun. 55: 2675-2680 [1987]; and F. Gunzer and H. Karch, "Expression of A and B subunits of Shiga-like toxin II as fusions with glutathione S-transferase and their potential for use in seroepidemiology.", J. Clin. Microbiol.. 31: 2604-2610 [1993]; and D.W. Acheson *et al.*, "Expression and purification of Shiga-like toxin II B subunits." Inf. Immun.. 63:301-308 [1995]). In one case, bench top fermentation techniques yielded 22 mg/liter of soluble recombinant protein (D. W. K. Acheson, *et al.*, "Comparison of Shiga-like toxin I B-subunit expression and localization in *Escherichia coli* and *Vibrio cholerae* by using *trc* or Iron-regulated promoter systems." Infect. Immun. 61: 1098-1104 [1993]). However, there have been no systematic approaches to identifying the appropriate spectrum of VT antigens, preserving immunogen and immunoabsorbant antigenicity and scaling-up.

The receptor for VT1 and VT2 is a globotriaosyl ceramide containing a galactose α -(1-4)- galactose- β -(1-4) glucose ceramide (Gb3) (C. A. Lingwood *et al.*, "Glycolipid binding of natural and recombinant *Escherichia coli* produced verotoxin *in vitro*," J. Biol. Chem.. 262: 1779-1785 [1987]; and T. Wadell *et al.*, "Globotriaosyl ceramide is specifically recognized by the *Escherichia coli* verocytotoxin 2." Biochem. Biophys. Res. Commun.. 152: 674-679 [1987]). Gb3 is abundant in the cortex of the human kidney and is present in primary human endothelial cell cultures. Hence, the identification of Gb3 as the functional receptor for VT1 and VT2 is consistent with their role in HUS pathogenesis, in which endothelial cells of the renal vasculature are the principal site of damage. Therefore, toxin-mediated pathogenesis may follow a sequence of B subunit binding to Gb3 receptors on kidney cells, toxin internalization, enzymatic reduction of the A subunit to an A1 fragment, binding of the A1 subunit to the 60S ribosomal subunit, inhibition of protein synthesis and cell death (A. D. O'Brien *et al.*, "Shiga and Shiga-like toxins. Microbial Rev.. 51: 206-220 [1987]).

The role of verotoxins in the pathogenesis of *E. coli* O157:H7 infections has been further studied in animal models. Infection or toxin challenge of laboratory animals do not produce all the pathologies and symptoms of hemorrhagic colitis, HUS, and TTP which occur in humans. Glomerular damage is noticeably absent. Nonetheless, experiments using animal models implicate verotoxins as the direct cause of hemorrhagic colitis, microvascular damage leading to the failure of kidneys and other organs and CNS neuropathies.

For example, Barrett, *et al.* delivered VT2 into the peritoneal cavity of rabbits using mini-osmotic pumps (J. J. Barrett *et al.*, "Continuous peritoneal infusion of shiga-like toxin II (SLTII) as a model for SLT II-induced diseases." *J. Infect. Dis.*, 159: 774-777 [1989]). In three days, most animals receiving the toxin developed diarrhea, with intestinal lesions resembling those seen in humans with hemorrhagic colitis. Although there was some evidence of renal dysfunction, none of the rabbits developed HUS. Beery, *et al.* showed that VT2, when administered intraperitoneally or intravenously to adult mice, produces lesions of the kidneys and colon (J. T. Beery *et al.*, "Cytotoxic activity of *Escherichia coli* O157:H7 culture filtrate on the mouse colon and kidney." *Curr. Microbiol.*, 11: 335-342 [1984]).

10 Histologic lesions in the kidney included accumulation of numerous exfoliated collecting tubules and marked intracellular vacuolation of proximal convoluted tubular cells.

Sjögren *et. al.* studied the pathogenesis of an entero-adherent strain of *E. coli* (RDEC-1) lysogenized with a VT1-containing bacteriophage (VT1-producing RDEC-1) (R. Sjögren *et al.*, "Role of Shiga-like toxin I in bacterial enteritis: comparison between isogenic 15 *Escherichia coli* strains induced in rabbits." *Gastroenterol.*, 106: 306-317 [1994]). In this study, rabbits were challenged with RDEC-1 or VT1-producing RDEC-1 and studied for onset of disease. The VT1-producing variant induced a severe, non-invasive, entero-adherent infection in rabbits which was characterized by serious histological lesions with vascular changes, edema and severe epithelial inflammation. Importantly, vascular changes consistent 20 with endothelial damage were seen in infected animals that was similar to intestinal microvascular changes in humans with *E. coli* O157:H7 infection. Based on these observations, they concluded that VT1 is an important virulence factor in enterohemorrhagic *E. coli* O157:H7 infection.

Fuji *et. al.* described a model in which mice were treated for three days with 25 streptomycin followed by a simultaneous challenge of *E. coli* O157:H7 orally, and mitomycin intraperitoneally (J. Fuji *et al.*, "Direct evidence of neuron impairment by oral infection with Verotoxin-producing *Escherichia coli* O157:H7 in mitomycin-treated mice." *Infect. Immun.*, 62: 3447-34453 [1994]). All of the animals died within four days. Immunoelectron-microscopy strongly suggested that death was due to the toxic effects of VT2v (a structural 30 variant of VT2), on both the endothelial cells and neurons in the central nervous system which resulted in fatal acute encephalopathy.

Wadolkowski *et al.* studied colonization of *E. coli* O157:H7 in mice. Mice were treated with streptomycin and fed 10^{10} *E. coli* O157:H7 (E. A. Wadolkowski *et al.*, "Mouse

model for colonization and disease caused by enterohemorrhagic *Escherichia coli* O157:H7." Infect. Immun. 58: 2438-2445 [1990]; and E. A. Wadolkowski *et al.*, "Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shiga-like toxin Type II." Infect. Immun. 58: 3959-3965 [1990]). All of the mice died due to 5 severe, disseminated, acute necrosis of proximal convoluted tubules. In mouse models, glomerular damage was not observed, but toxic acute renal tubular necrosis was observed which is characteristic of some HUS patients. The failure of mice to show glomerular damage is thought to be due to the absence of a functional globotriaosyl ceramide receptor specific for verotoxins in the glomeruli of the kidneys. Administration of VT2 subunit- 10 specific monoclonal antibodies prior to infection prevented all pathology and death.

E. Current Therapeutic Approaches

E. coli O157:H7 disease is not adequately controlled by current therapy. Patient treatment is tailored to manage fluid and electrolyte disturbances, anemia, renal failure and hypertension. Although *E. coli* O157:H7 is susceptible to common antibiotics, the role of 15 antibiotics in the treatment of infection has questionable merit. In both retrospective and prospective studies, prophylaxis or treatment with antibiotics such as trimethoprim-sulfamethoxazole, there was either no benefit or an increased risk of developing HUS (T. N. Bokete *et al.*, "Shiga-like toxin producing *Escherichia coli* in Seattle children: a prospective study." Gastroenterol. 105: 1724-1731 [1993]; A. T. Pavia *et al.*, "Hemolytic uremic- 20 syndrome during an outbreak of *Escherichia coli* O157:H7 infections in institutions for mentally retarded persons: clinical and epidemiologic observations." J. Pediatr. 116: 544-551 [1990]; F. Proulx *et al.*, "Randomized, controlled trial of antibiotic therapy for *Escherichia coli* O157:H7 enteritis." J. Pediatr. 121: 299-303 [1992]; and A. L. Carter *et al.*, "A severe 25 outbreak of *Escherichia coli* O157:H7-associated hemorrhagic colitis in a nursing home." New Eng. J. Med., 317: 1496-1500 [1987]).

The mechanisms by which antibiotics increase the risk of infection or related complications might involve enhancement of toxin production, release of toxins from killed organisms, or alteration of normal competing intestinal flora allowing for pathogen overgrowth (M. A. Karmali, "Infection by Verocytotoxin-producing *Escherichia coli*." Clin. 30 Microbiol. Rev., 2: 15-38 [1989]). A further concern in the use of antibiotics is the potential acquisition of antimicrobial resistance by *E. coli* O157:H7 (C. R. Dorn, "Review of foodborne outbreak of *Escherichia coli* O157:H7 infection in the western United States." JAVMA 203: 1583-1587 [1993]).

In addition, by the time symptoms are serious enough to attract medical attention, it is likely that verotoxins are already entering the systemic circulation or will do so shortly thereafter. Although antimicrobials may help to prevent pathology resulting from the action of toxin on the bowel lumen. However, by the time symptoms of HUS have developed, the 5 patient has ceased shedding organisms. Thus, antimicrobial treatment during HUS disease is of less value, and often contraindicated, due to the increased risk of complications associated with administration of antimicrobials to patients susceptible to development of HUS. Importantly, there is currently no antitoxin commercially available for use in treating affected 10 patients. What is needed is a means to block the progression of disease, without the complications associated with antimicrobial treatment.

DESCRIPTION OF THE DRAWINGS

Figure 1 is an SDS-PAGE of rVT1 and rVT2.

Figure 2 shows HPLC results for rVT1 and rVT2.

15 Figure 3 shows rVT1 and rVT2 toxicity in Vero cell culture.

Figure 4 shows EIA reactivity of rVT1 and rVT2 antibodies to rVT1.

Figure 5 shows EIA reactivity of rVT1 and rVT2 Antibodies to rVT2.

Figure 6 shows Western Blot reactivity of rVT1 and rVT2 antibodies to rVT's:

20 Panel 6A contains preimmune IgY;

Panel 6B contains rVT1 IgY; and

Panel 6C contains rVT2 IgY.

Figure 7 shows neutralization of rVT1 cytotoxicity in Vero cells.

Figure 8 shows neutralization of rVT2 cytotoxicity in Vero cells.

25 Figure 9 shows renal sections from *E. coli* O157:H7-infected mice treated with IgY

Panel 9A shows a representative kidney section from a mouse treated with preimmune IgY;

Panel 9B shows a representative kidney sections from a mouse treated with rVT1; and

Panel 9C shows a representative kidney section from a mouse treated with rVT2 IgY.

30 Figure 10 shows the fusion constructs of VT components and affinity tags.

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

As used herein, the term "neutralizing" is used in reference to antitoxins, particularly antitoxins comprising antibodies, which have the ability to prevent the pathological actions of the toxin against which the antitoxin is directed.

As used herein, the term "overproducing" is used in reference to the production of toxin polypeptides in a host cell, and indicates that the host cell is producing more of the toxin by virtue of the introduction of nucleic acid sequences encoding the toxin polypeptide than would be expressed by the host cell absent the introduction of these nucleic acid sequences. To allow ease of purification of toxin polypeptides produced in a host cell it is preferred that the host cell express or overproduce the toxin polypeptide at a level greater than 1 mg/liter of host cell culture.

As used herein, the term "fusion protein" refers to a chimeric protein containing the protein of interest (*i.e.*, an *E. coli* verotoxin and/or fragments thereof) joined to an exogenous protein fragment (the fusion partner which consists of a non-toxin protein). The fusion partner may enhance solubility of the *E. coli* protein as expressed in a host cell, may provide an "affinity tag" to allow purification of the recombinant fusion protein from the host cell or culture supernatant, or both. If desired, the fusion protein may be removed from the protein of interest (*i.e.*, toxin protein or fragments thereof) prior to immunization by a variety of enzymatic or chemical means known to the art.

As used herein, the term "affinity tag" refers to such structures as a "poly-histidine tract" or "poly-histidine tag," or any other structure or compound which facilitates the purification of a recombinant fusion protein from a host cell, host cell culture supernatant, or both. As used herein, the term "flag tag" refers to short polypeptide marker sequence useful for recombinant protein identification and purification.

As used herein, the terms "poly-histidine tract" and "poly-histidine tag," when used in reference to a fusion protein refers to the presence of two to ten histidine residues at either the amino- or carboxy-terminus of a protein of interest. A poly-histidine tract of six to ten residues is preferred. The poly-histidine tract is also defined functionally as being a number of consecutive histidine residues added to the protein of interest which allows the affinity purification of the resulting fusion protein on a nickel-chelate column.

As used herein, the term "chimeric protein" refers to two or more coding sequences obtained from different genes, that have been cloned together and that, after translation, act as

a single polypeptide sequence. Chimeric proteins are also referred to as "hybrid proteins." As used herein, the term "chimeric protein" refers to coding sequences that are obtained from different species of organisms, as well as coding sequences that are obtained from the same species of organisms.

5 As used herein, the term "protein of interest" refers to the protein whose expression is desired within the fusion protein. In a fusion protein, the protein of interest will be joined or fused with another protein or protein domain, the fusion partner, to allow for enhanced stability of the protein of interest and/or ease of purification of the fusion protein.

10 As used herein, the term "maltose binding protein" and "MBP" refers to the maltose binding protein of *E. coli*. A portion of the maltose binding protein may be added to a protein of interest to generate a fusion protein: a portion of the maltose binding protein may merely enhance the solubility of the resulting fusion protein when expressed in a bacterial host. On the other hand, a portion of the maltose binding protein may allow affinity purification of the fusion protein on an amylose resin.

15 As used herein, the term "purified" or "to purify" refers to the removal of contaminants from a sample. For example, antitoxins are purified by removal of contaminating non-immunoglobulin proteins: they are also purified by the removal of substantially all immunoglobulin that does not bind toxin. The removal of non-immunoglobulin proteins and/or the removal of immunoglobulins that do not bind toxin 20 results in an increase in the percent of toxin-reactive immunoglobulins in the sample. In another example, recombinant toxin polypeptides are expressed in bacterial host cells and the toxin polypeptides are purified by the removal of host cell proteins: the percent of recombinant toxin polypeptides is thereby increased in the sample.

25 The term "recombinant DNA molecule" as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques.

The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed from a recombinant DNA molecule.

30 The term "native protein" as used herein refers to a protein which is isolated from a natural source as opposed to the production of a protein by recombinant means.

As used herein the term "portion" when in reference to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid.

As used herein "soluble" when in reference to a protein produced by recombinant DNA technology in a host cell, is a protein which exists in solution in the cytoplasm of the host cell; if the protein contains a signal sequence, the soluble protein is exported to the periplasmic space in bacterial hosts and is secreted into the culture medium of eukaryotic cells 5 capable of secretion or by bacterial hosts possessing the appropriate genes. In contrast, an insoluble protein is one which exists in denatured form inside cytoplasmic granules (called an inclusion bodies) in the host cell. High level expression (*i.e.*, greater than 1 mg recombinant protein/liter of bacterial culture) of recombinant proteins often results in the expressed protein being found in inclusion bodies in the bacterial host cells. A soluble protein is a protein 10 which is not found in an inclusion body inside the host cell or is found both in the cytoplasm and in inclusion bodies and in this case the protein may be present at high or low levels in the cytoplasm.

A distinction is drawn between a soluble protein (*i.e.*, a protein which when expressed in a host cell is produced in a soluble form) and a "solubilized" protein. An insoluble 15 recombinant protein found inside an inclusion body may be solubilized (*i.e.*, rendered into a soluble form) by treating purified inclusion bodies with denaturants such as guanidine hydrochloride, urea or sodium dodecyl sulfate (SDS). These denaturants must then be removed from the solubilized protein preparation to allow the recovered protein to renature (refold). Not all proteins will refold into an active conformation after solubilization in a 20 denaturant and removal of the denaturant. Many proteins precipitate upon removal of the denaturant. SDS may be used to solubilize inclusion bodies and will maintain the proteins in solution at low concentration. However, dialysis will not always remove all of the SDS (SDS can form micelles which do not dialyze out); therefore, SDS-solubilized inclusion body protein is soluble but not refolded.

25 As used herein, the term "reporter reagent" or "reporter molecule" is used in reference to compounds which are capable of detecting the presence of antibody bound to antigen. For example, a reporter reagent may be a colorimetric substance which is attached to an enzymatic substrate. Upon binding of antibody and antigen, the enzyme acts on its substrate and causes the production of a color. Other reporter reagents include, but are not limited to 30 fluorogenic and radioactive compounds or molecules.

As used herein the term "signal" is used in reference to the production of a sign that a reaction has occurred, for example, binding of antibody to antigen. It is contemplated that signals in the form of radioactivity, fluorogenic reactions, and enzymatic reactions will be

used with the present invention. The signal may be assessed quantitatively as well as qualitatively.

As used herein, the term "therapeutic amount" refers to that amount of antitoxin required to neutralize the pathologic effects of *E. coli* toxin in a subject.

5 As used herein, the term "acute intoxication" is used in reference to cases of *E. coli* infection in which the patient is currently suffering from the effects of toxin (e.g., *E. coli* verotoxins or enterotoxins). Signs and symptoms of intoxication with the toxin may be immediately apparent. Or, the determination of intoxication may require additional testing, such as detection of toxin present in the patient's fecal material.

10 As used herein, the term "at risk" is used in references to individuals who have been exposed to *E. coli* and may suffer the symptoms associated with infection or disease with these organisms, especially due to the effects of verotoxins.

SUMMARY OF THE INVENTION

15 The present invention relates to antitoxin therapy for humans and other animals. Antitoxins which neutralize the pathologic effects of *E. coli* toxins are generated by immunization of avian hosts with recombinant toxin fragments. In one embodiment, the present invention contemplates a method of treatment administering at least one antitoxin directed against at least a portion of an *Escherichia coli* verotoxin in an aqueous solution in 20 therapeutic amount that is administrable to an intoxicated subject. It is contemplated that the intoxicated subject will be either an adult or a child.

25 In a preferred embodiment, the *E. coli* verotoxin is recombinant. In one embodiment, the antitoxin is an avian antitoxin. In an alternative embodiment, the recombinant *E. coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT1 sequence. In one embodiment of the *E. coli* fusion protein, the fusion protein comprises a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT2 sequence.

30 Various routes of administration, are contemplated for providing the *E. coli* antitoxin(s) to an affected individual, including but not limited to, parenteral as well as oral routes of administration. In a particularly preferred embodiment, the route of administration is parenteral.

The present invention also includes the embodiment of a method of prophylactic treatment in which an antitoxin directed against at least one *E. coli* verotoxin in an aqueous

solution in therapeutic amount that is parenterally administrable, and is administered to at least one subject at risk of diarrheal disease. In one embodiment, the antitoxin is parenterally administered.

5 In one embodiment, the subject is at risk of developing extra-intestinal complications of *E. coli* infections, including but not limited to, hemolytic uremic syndrome, thrombotic thrombocytopenic purpura, etc.

The present invention also includes the embodiment of a composition which comprises neutralizing antitoxin directed against at least one *E. coli* verotoxin in an aqueous solution in therapeutic amounts. In one particularly preferred embodiment, the *E. coli* verotoxin is a recombinant toxin. In an alternative embodiment, the recombinant *E. coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *E. coli* verotoxin VT1 sequence. In another embodiment, the recombinant *E. coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *E. coli* verotoxin VT2 sequence. In yet another embodiment, the composition of the antitoxin is directed against a portion of at least one *Escherichia coli* verotoxin. In one embodiment, the portion of *Escherichia coli* is selected from the group consisting of subunit A and subunit B of VT1. In an alternative embodiment, the portion of *Escherichia coli* is selected from the group consisting of subunit A and subunit B of VT2. Indeed, the invention contemplates an antitoxin that is directed against a portion of at least one *Escherichia coli* verotoxin. In one embodiment, the antitoxin is an avian antitoxin.

20 The present invention also comprises a method of treatment of enteric bacterial infections comprising administering an avian antitoxin directed against at least one verotoxin produced by *E. coli* in an aqueous solution in therapeutic amount, to at least one infected subject. In one preferred embodiment, the avian antitoxin is administered parenterally.

25 In another embodiment, the *E. coli* is selected from the group consisting of *Escherichia coli* serotypes O157:H7, O1:NM; O2:H5; O2:H7; O4:NM; O4:H10; O5:NM; O5:H16; O6:H1; O18:NM; O18:H7; O25:NM; O26:NM; O26:H11; O26:H32; O38:H21; O39:H4; O45:H2; O50:H7; O55:H7; O55:H10; O82:H8; O84:H2; O91:NM; O91:H21; O103:H2; O111:NM; O111:H8; O111:H30; O111:H34; O113:H7; O113:H21; O114:H48; O115:H10; O117:H4; O118:H12; O118:H30; O121:NM; O121:H19; O125:NM; O125:H8; O126:NM; O126:H8; O128:NM; O128:H2; O128:H8; O128:H12; O128:H25; O145:NM; O125:H25; O146:H21; O153:H25; O157:NM; O163:H19; O165:NM; O165:19; and O165:H25. In one embodiment, the antitoxin comprises antitoxin directed against at least one

Escherichia coli verotoxin. In another embodiment, the antitoxin is cross-reacts with at least one *Escherichia coli* verotoxin. In yet another embodiment, the antitoxin is reactive against toxins produced by members of the genus *Shigella*, including *S. dysenteriae*.

The present invention also contemplates uses for the toxin fragments in vaccines and 5 diagnostic assays. The fragments may be used separately as purified, soluble antigens or, alternatively, in mixtures or "cocktails." The present invention thus comprises a method for detecting *Escherichia coli* verotoxin in a sample in which a sample, an antitoxin raised against *Escherichia coli* verotoxin, and a reporter reagent capable of binding the antitoxin are provided. The antitoxin is added to the sample, so that the antitoxin binds to the *E. coli* 10 verotoxin in the sample. In one embodiment, the antitoxin is an avian antitoxin. In an alternative embodiment, the method further comprises the steps of washing unbound antitoxin from the sample, adding at least one reporter reagent to the sample, so that said reporter reagent binds to any antitoxin that is bound, washing the unbound reporter reagent from the sample and detecting the reporter reagent bound to the antitoxin bound to the *Escherichia coli* 15 verotoxin, so that the verotoxin is detected. In one embodiment, the detecting is accomplished through any means, such as enzyme immunoassay, radioimmunoassay, fluorescence immunoassay, flocculation, particle agglutination, and *in situ* chromogenic assay. In one preferred embodiment, the sample is a biological sample. In an alternative preferred embodiment, the sample is an environmental sample.

20

DESCRIPTION OF THE INVENTION

The present invention contemplates treating humans and other animals intoxicated with at least one bacterial toxin. It is contemplated that administration of antitoxin will be used to treat patients effected by or at risk of symptoms due to the action of bacterial toxins. It is 25 also contemplated that the antitoxin will be used in a diagnostic assay to detect the presence of toxins in samples. The organisms, toxins and individual steps of the present invention are described separately below.

I. Antibodies Directed Against *E. coli* and Associated Toxins

30

A preferred embodiment of the method of the present invention is directed toward obtaining antibodies against various *E. coli* serotypes, their toxins, enzymes or other metabolic by-products, cell wall components, or synthetic or recombinant versions of any of these compounds. It is contemplated that these antibodies will be produced by immunization

of humans or other animals. It is not intended that the present invention be limited to any particular toxin or any species of organism. In one embodiment, toxins from all *E. coli* serotypes are contemplated as immunogens. Examples of these toxins include the verotoxins VT1 and VT2.

5 It is not intended that antibodies produced against one toxin will only be used against that toxin. It is contemplated that antibodies directed against one toxin may be used as an effective therapeutic against one or more toxin(s) produced by other *E. coli* serotypes, or other toxin producing organisms (e.g., *Shigella*, *Bacillus cereus*, *Staphylococcus aureus*, *Streptococcus mutans*, *Acinetobacter calcoaceticus*, *Pseudomonas aeruginosa*, other 10 *Pseudomonas* species, *Vibrio* species, *Clostridium* species, etc.). It is further contemplated that antibodies directed against the portion of the toxin which binds to mammalian membranes can also be used against other organisms. It is contemplated that these membrane binding domains are produced synthetically and used as immunogens.

15 **II. Obtaining Antibodies In Non-Mammals**

A preferred embodiment of the method of the present invention for obtaining antibodies involves immunization. However, it is also contemplated that antibodies may be obtained from non-mammals without immunization. In the case where no immunization is contemplated, the present invention may use non-mammals with preexisting antibodies to 20 toxins as well as non-mammals that have antibodies to whole organisms by virtue of reactions with the administered antigen. An example of the latter involves immunization with synthetic peptides or recombinant proteins sharing epitopes with whole organism components.

In a preferred embodiment, the method of the present invention contemplates 25 immunizing non-mammals with bacterial toxin(s). It is not intended that the present invention be limited to any particular toxin. In one embodiment, toxins from all *E. coli* serotypes are contemplated as immunogens.

A particularly preferred embodiment involves the use of bacterial toxin protein or 30 fragments of toxin proteins produced by molecular biological means (i.e., recombinant toxin proteins). In a preferred embodiment, the immunogen comprises recombinant VT1 and/or VT2.

When immunization is used, the preferred non-mammal is from the class *Aves*. All birds are contemplated (e.g., duck, ostrich, emu, turkey, etc.). A preferred bird is a chicken. Importantly, chicken antibody does not fix mammalian complement (See H.N. Benson *et al.*,

J. Immunol. 87:616 [1961]). Thus, chicken antibody will normally not cause a complement-dependent reaction (A.A. Benedict and K. Yamaga. "Immunoglobulins and Antibody Production in Avian Species," in *Comparative Immunology* (J.J. Marchaloni, ed.), pp. 335-375. Blackwell, Oxford [1966]). Thus, the preferred antitoxins of the present invention will 5 not exhibit complement-related side effects observed with antitoxins presently known.

When birds are used, it is contemplated that the antibody will be obtained from either the bird serum or the egg. A preferred embodiment involves collection of the antibody from the egg. Laying hens transport immunoglobulin to the egg yolk ("IgY") in concentrations equal to or exceeding that found in serum (See R. Patterson *et al.*, J. Immunol. 89:272 10 (1962); and S.B. Carroll and B.D. Stollar, J. Biol. Chem. 258:24 [1983]). In addition, the large volume of egg yolk produced vastly exceeds the volume of serum that can be safely obtained from the bird over any given time period. Finally, the antibody from eggs is more pure and more homogeneous: there is far less non-immunoglobulin protein (as compared to serum) and only one class of immunoglobulin is transported to the yolk.

15 When considering immunization with toxins, one may consider modification of the toxins to reduce the toxicity. In this regard, it is not intended that the present invention be limited by immunization with modified toxin. Unmodified ("native") toxin is also contemplated as an immunogen.

It is also not intended that the present invention be limited by the type of modification 20 -- if modification is used. The present invention contemplates all types of toxin modification, including chemical and heat treatment of the toxin. In one embodiment, glutaraldehyde treatment of the toxin is contemplated. In an alternative embodiment, formaldehyde treatment of the toxin is contemplated.

25 It is not intended that the present invention be limited to a particular mode of immunization: the present invention contemplates all modes of immunization, including subcutaneous, intramuscular, intraperitoneal, and intravenous or intravascular injection, as well as *per os* administration of immunogen.

The present invention further contemplates immunization with or without adjuvant. As 30 used herein, the term "adjuvant" is defined as a substance known to increase the immune response to other antigens when administered with other antigens. If adjuvant is used, it is not intended that the present invention be limited to any particular type of adjuvant -- or that the same adjuvant, once used, be used all the time. While the present invention contemplates all types of adjuvant, whether used separately or in combinations, the preferred use of

adjvant is the use of Complete Freund's Adjuvant followed sometime later with Incomplete Freund's Adjuvant. The invention also contemplates the use of fowl adjuvant commercially available from RIBI, as well as Quil A adjuvant commercially available from Accurate Chemical and Scientific Corporation, and Gerbu adjuvant also commercially available (GmDP: C.C. Biotech Corp.).

When immunization is used, the present invention contemplates a wide variety of immunization schedules. In one embodiment, a chicken is administered toxin(s) on day zero and subsequently receives toxin(s) in intervals thereafter. It is not intended that the present invention be limited by the particular intervals or doses. Similarly, it is not intended that the present invention be limited to any particular schedule for collecting antibody. The preferred collection time is sometime after day 35.

Where birds are used and collection of antibody is performed by collecting eggs, the eggs may be stored prior to processing for antibody. It is preferred that eggs be stored at 4°C for less than one year.

It is contemplated that chicken antibody produced in this manner can be buffer-extracted and used analytically. While unpurified, this preparation can serve as a reference for activity of the antibody prior to further manipulations (e.g., immunoaffinity purification).

III. Increasing The Effectiveness Of Antibodies

When purification is used, the present invention contemplates purifying to increase the effectiveness of both non-mammalian antitoxins and mammalian antitoxins. Specifically, the present invention contemplates increasing the percent of toxin-reactive immunoglobulin. The preferred purification approach for avian antibody is polyethylene glycol (PEG) separation.

The present invention contemplates that avian antibody be initially purified using simple, inexpensive procedures. In one embodiment, chicken antibody from eggs is purified by extraction and precipitation with PEG. PEG purification exploits the differential solubility of lipids (which are abundant in egg yolks) and yolk proteins in high concentrations of PEG 8000 (Polson *et al.*, Immunol. Comm. 9:495 [1980]). The technique is rapid, simple, and relatively inexpensive and yields an immunoglobulin fraction that is significantly more pure, in terms of contaminating non-immunoglobulin proteins than the comparable ammonium sulfate fractions of mammalian sera and horse antibodies. The majority of the PEG is removed from the precipitated chicken immunoglobulin by treatment with ethanol. Indeed,

PEG-purified antibody is sufficiently pure that the present invention contemplates the use of PEG-purified antitoxins in the passive immunization of intoxicated humans and animals.

IV. Treatment

5 The present invention contemplates antitoxin therapy for humans and other animals intoxicated by bacterial toxins. A preferred method of treatment is by parenteral administration of antitoxin.

A. Dosage Of Antitoxin

10 It was noted by way of background that a balance must be struck when administering currently available antitoxin which is usually produced in large animals such as horses: sufficient antitoxin must be administered to neutralize the toxin, but not so much antitoxin as to increase the risk of untoward side effects. These side effects are caused by: i) patient sensitivity to foreign (e.g., horse) proteins; ii) anaphylactic or immunogenic properties of non-immunoglobulin proteins; iii) the complement fixing properties of mammalian antibodies; and/or iv) the overall burden of foreign protein administered. It is extremely difficult to strike this balance when, as noted above, the degree of intoxication (and hence the level of antitoxin therapy needed) can only be approximated.

20 The present invention contemplates significantly reducing side effects so that this balance is more easily achieved. Treatment according to the present invention contemplates reducing side effects by using PEG-purified antitoxin from birds.

25 In one embodiment, the treatment of the present invention contemplates the use of PEG-purified antitoxin from birds. The use of yolk-derived, PEG-purified antibody as antitoxin allows for the administration of: 1) non (mammalian)-complement-fixing, avian antibody; 2) a less heterogeneous mixture of non-immunoglobulin proteins; and 3) less total protein to deliver the equivalent weight of active antibody present in currently available antitoxins. The non-mammalian source of the antitoxin makes it useful for treating patients who are sensitive to horse or other mammalian sera.

30 As is true in cases of botulism, the degree of an individual's exposure to *E. coli* toxin and the prognosis are often difficult to assess, and depend upon a number of factors (e.g., the quantity of contaminated food ingested, the toxigenicity and serotype of *E. coli* strain ingested, etc.). Thus, the clinical presentation of a patient is usually a more important consideration than a quantitative diagnostic test, for determination of dosage in antitoxin

administration. Indeed, for many toxin-associated diseases (e.g., botulism, tetanus, diphtheria, etc.), there is no rapid, quantitative test to detect the presence of the toxin or organism. Rather, these toxin-associated diseases are medical emergencies which mandate immediate treatment. Confirmation of the etiologic agent must not delay the institution of therapy, as the condition of an affected patient may rapidly deteriorate. In addition to the initial treatment with antitoxin, subsequent doses may be indicated, as the patient's disease progresses. The dosage and timing of these subsequent doses is dependent upon the signs and symptoms of disease in each individual patient.

It is contemplated that the administration of antitoxin to an affected individual would involve an initial injection of an approximately 10 ml dose of immune globulin (with less than approximately 1 gram of total protein). In one preferred embodiment, it is contemplated that at least 50% of the initial injection comprises immune globulin. It is also contemplated that more purified immune globulin be used for treatment, wherein approximately 90% of the initial injection comprises immune globulin. When more purified immune globulin is used, it is contemplated that the total protein will be less than approximately 100 milligrams. It is also contemplated that additional doses be given, depending upon the signs and symptoms associated with *E. coli* verotoxin disease progression.

B. Delivery Of Antitoxin

Although it is not intended to limit the route of delivery, the present invention contemplates a method for antitoxin treatment of bacterial intoxication in which delivery of antitoxin is parenteral or oral.

In one embodiment, antitoxin is parenterally administered to a subject in an aqueous solution. It is not intended that the parenteral administration be limited to a particular route. Indeed, it is contemplated that all routes of parenteral administration will be used. In one embodiment, parenteral administration is accomplished via intramuscular injection. In an alternative embodiment, parenteral administration is accomplished via intravenous injection.

In another embodiment, antitoxin is delivered in a solid form (e.g., tablets). In an alternative embodiment antitoxin is delivered in an aqueous solution. When an aqueous solution is used, the solution has sufficient ionic strength to solubilize antibody protein, yet is made palatable for oral administration. The delivery solution may also be buffered (e.g., carbonate buffer, pH 9.5) which can neutralize stomach acids and stabilize the antibodies when the antibodies are administered orally. In one embodiment the delivery solution is an

aqueous solution. In another embodiment the delivery solution is a nutritional formula. Preferably, the delivery solution is infant or a dietary supplement formula (e.g., Similac®, Ensure®, and Enfamil®). Yet another embodiment contemplates the delivery of lyophilized antibody encapsulated or microencapsulated inside acid-resistant compounds.

5 Methods of applying enteric coatings to pharmaceutical compounds are well known to the art (companies specializing in the coating of pharmaceutical compounds are available: for example, The Coating Place [Verona, WI] and AAI [Wilmington, NC]). Enteric coatings which are resistant to gastric fluid and whose release (*i.e.*, dissolution of the coating to release the pharmaceutical compound) is pH dependent are commercially available (for example, the polymethacrylates Eudragit® L and Eudragit® S [Röhm Tech Inc., Malden, MA]).

10 Eudragit® S is soluble in intestinal fluid from pH 7.0; this coating can be used to microencapsulate lyophilized antitoxin antibodies and the particles are suspended in a solution having a pH above or below pH 7.0 for oral administration. The microparticles will remain intact and undissolved until they reached the intestines where the intestinal pH would cause 15 them to dissolve thereby releasing the antitoxin.

20 The invention contemplates a method of treatment which can be administered for treatment of acute intoxication. In one embodiment, antitoxin is administered orally in either a delivery solution or in tablet form, in therapeutic dosage, to a subject intoxicated by the bacterial toxin which served as immunogen for the antitoxin. In another embodiment of treatment of acute intoxication, a therapeutic dosage of the antitoxin in a delivery solution, is 25 parenterally administered.

25 The invention also contemplates a method of treatment which can be administered prophylactically. In one embodiment, antitoxin is administered orally, in a delivery solution, in therapeutic dosage, to a subject, to prevent intoxication of the subject by the bacterial toxin which served as immunogen for the production of antitoxin. In another embodiment, antitoxin is administered orally in solid form such as tablets or as microencapsulated particles. Microencapsulation of lyophilized antibody using compounds such as Eudragit® (Rohm 30 GmbH) or polyethylene glycol, which dissolve at a wide range of pH units, allows the oral administration of solid antitoxin in a liquid form (*i.e.*, a suspension) to recipients unable to tolerate administration of tablets (e.g., children or patients on feeding tubes). In one preferred embodiment the subject is a child. In another embodiment, antibody raised against whole bacterial organism is administered orally to a subject, in a delivery solution, in therapeutic

dosage. In yet another preferred embodiment of prophylactic treatment, a therapeutic dosage of the antitoxin in a delivery solution, is parenterally administered.

V. Multivalent Vaccines Against *E. coli* Strains

5 The invention contemplates the generation of multivalent vaccines for the protection of an organism (particularly humans) against several *E. coli* strains. Of particular interest is a vaccine which stimulates the production of a humoral immune response to *E. coli* O157:H7, O26:H11, O113:H21, O91:H21, and O111:NM, in humans. The antigens comprising the vaccine preparation may be native or recombinantly produced toxin proteins from the *E. coli* 10 serotypes listed above. When native toxin proteins are used as immunogens they are generally modified to reduce the toxicity. It is contemplated that glutaraldehyde-modified toxin proteins will be used. In an alternative embodiment, formaldehyde-modified toxin proteins will be used.

15 The invention contemplates that recombinant *E. coli* verotoxin proteins be used in conjunction with either native toxins or toxoids from other organisms as antigens in a multivalent vaccine preparation. It is also contemplated that recombinant *E. coli* toxin proteins be used in the multivalent vaccine preparation.

VI. Detection Of Toxin

20 The invention contemplates detecting bacterial toxin in a sample. The term "sample" in the present specification and claims is used in its broadest sense. On the one hand it is meant to include a specimen or culture (e.g., microbiological cultures). On the other hand, it is meant to include both biological and environmental samples.

25 Biological samples may be animal, including human, fluid, solid (e.g., stool) or tissue, as well as liquid and solid food and feed products and ingredients such as dairy items, vegetables, meat and meat by-products, and waste. Biological samples may be obtained from all of the various families of common domestic animals, including but not limited, to bovines (e.g. cattle), ovines (e.g., sheep), caprines (e.g., goats), porcines (e.g., swine), equines (e.g., horses), canines (e.g., dogs), lagamorphs (e.g., rabbits), and felines (e.g., cats), etc. It is also intended that samples may be obtained from feral or wild animals, including, but not limited to, such animals as ungulates (e.g., deer), bear, fish, lagamorphs, rodents, etc.

30 Environmental samples include environmental material such as surface matter, soil, water and industrial samples, as well as samples obtained from food and dairy processing

instruments, apparatus, equipment, utensils, disposable and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention.

The invention contemplates detecting bacterial toxin by a competitive immunoassay 5 method that utilizes recombinant toxin VT1 and toxin VT2 proteins, antibodies raised against recombinant bacterial toxin proteins. A fixed amount of the recombinant toxin proteins are immobilized to a solid support (e.g., a microtiter plate) followed by the addition of a biological sample suspected of containing a bacterial toxin. The biological sample is first mixed with affinity-purified or PEG fractionated antibodies directed against the recombinant 10 toxin protein. A reporter reagent is then added which is capable of detecting the presence of antibody bound to the immobilized toxin protein. The reporter substance may comprise an antibody with binding specificity for the antitoxin attached to a molecule which is used to identify the presence of the reporter substance. If toxin is present in the sample, this toxin will compete with the immobilized recombinant toxin protein for binding to the anti- 15 recombinant antibody thereby reducing the signal obtained following the addition of the reporter reagent. A control is employed where the antibody is not mixed with the sample. This gives the highest (or reference) signal.

The invention also contemplates detecting bacterial toxin by a "sandwich" immunoassay method that utilizes antibodies directed against recombinant bacterial toxin 20 proteins. Affinity-purified antibodies directed against recombinant bacterial toxin proteins are immobilized to a solid support (e.g., microtiter plates). Biological samples suspected of containing bacterial toxins are then added followed by a washing step to remove substantially all unbound antitoxin. The biological sample is next exposed to the reporter substance, which binds to antitoxin and is then washed free of substantially all unbound reporter substance. 25 The reporter substance may comprise an antibody with binding specificity for the antitoxin attached to a molecule which is used to identify the presence of the reporter substance. Identification of the reporter substance in the biological tissue indicates the presence of the bacterial toxin.

It is also contemplated that bacterial toxin be detected by pouring liquids (e.g., soups 30 and other fluid foods and feeds including nutritional supplements for humans and other animals) over immobilized antibody which is directed against the bacterial toxin. It is contemplated that the immobilized antibody will be present in or on such supports as cartridges, columns, beads, or any other solid support medium. In one embodiment, following

the exposure of the liquid to the immobilized antibody, unbound toxin is substantially removed by washing. The liquid is then exposed to a reporter substance which detects the presence of bound toxin. In a preferred embodiment the reporter substance is an enzyme, fluorescent dye, or radioactive compound attached to an antibody which is directed against the toxin (*i.e.*, in a "sandwich" immunoassay). It is also contemplated that the detection system will be developed as necessary (*e.g.*, the addition of enzyme substrate in enzyme systems; observation using fluorescent light for fluorescent dye systems; and quantitation of radioactivity for radioactive systems).

10 EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the disclosure which follows, the following abbreviations apply: °C (degrees Centigrade); rpm (revolutions per minute); BSA (bovine serum albumin); ELISA (enzyme-linked immunosorbent assay); IgG (immunoglobulin G); IgY (immunoglobulin Y); IP (intraperitoneal); SC (subcutaneous); H₂O (water); HCl (hydrochloric acid); LD₁₀₀ (lethal dose for 100% of experimental animals); aa (amino acid); HPLC (high performance liquid chromatography); Kda (kilodaltons); gm (grams); µg (micrograms); mg (milligrams); ng (nanograms); µl (microliters); ml (milliliters); mm (millimeters); nm (nanometers); µm (micrometer); M (molar); mM (millimolar); MW (molecular weight); sec (seconds); min(s) (minute/minutes); hr(s) (hour/hours); MgCl₂ (magnesium chloride); NaCl (sodium chloride); Na₂CO₃ (sodium carbonate); OD₂₈₀ (optical density at 280 nm); OD₆₀₀ (optical density at 600 nm); PAGE (polyacrylamide gel electrophoresis); PBS [phosphate buffered saline (150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2)]; PEG (polyethylene glycol); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl)aminomethane); w/v (weight to volume); v/v (volume to volume); Amicon (Amicon, Inc., Beverly, MA); Amresco (Amresco, Inc., Solon, OH); ATCC (American Type Culture Collection, Rockville, MD); BBL (Baltimore Biologics Laboratory, (a division of Becton Dickinson), Cockeysville, MD); Becton Dickinson (Becton Dickinson Labware, Lincoln Park, NJ); BioRad (BioRad, Richmond, CA); Biotech (C-C Biotech Corp., Poway, CA); Charles River (Charles River Laboratories, Wilmington, MA); Falcon (*e.g.* Baxter Healthcare Corp., McGaw Park, IL and Becton Dickinson); Fisher Biotech (Fisher Biotech, Springfield, NJ); GIBCO (Grand Island Biologic Company/BRL, Grand Island, NY); Mallinckrodt (a division of Baxter Healthcare Corp., McGaw Park, IL);

Millipore (Millipore Corp., Marlborough, MA); New England Biolabs (New England Biolabs, Inc., Beverly, MA); Novagen (Novagen, Inc., Madison, WI); Pharmacia (Pharmacia, Inc., Piscataway, NJ); Qiagen (Qiagen, Chatsworth, CA); Showdex (Showa Denko America, Inc., New York, NY); Sigma (Sigma Chemical Co., St. Louis, MO); RIBI (RIBI Immunochemical Research Inc., Hamilton, MT); Accurate Chemical and Scientific Corp. (Accurate Chemical and Scientific Corp., Hicksville, NY); Kodak (Eastman-Kodak, Rochester, NY); and Stratagene (Stratagene, La Jolla, CA).

When a recombinant protein is described in the specification it is referred to in a short-hand manner by the amino acids in the toxin sequence present in the recombinant protein rounded to the nearest 10. The specification gives detailed construction details for all recombinant proteins such that one skilled in the art will know precisely which amino acids are present in a given recombinant protein.

The first set of Examples (Examples 1-5) was designed to develop an antidote to *E. coli* O157:H7 verotoxins and evaluate its effectiveness *in vitro* and *in vivo*. In the first experiments, high titer verotoxin antibodies were generated in laying hens hyperimmunized with chemically detoxified and/or native verotoxins. These Laying hens were immunized with either recombinant *E. coli* O157:H7 VT1 or VT2 (rVT1 and rVT2) treated with glutaraldehyde and mixed with adjuvant.

Next, toxin-reactive polyclonal antibodies were isolated by bulk fractionation from egg yolks pooled from hyperimmunized hens. Large quantities of polyclonal antibodies (IgY) were harvested from resulting eggs using a two-step polyethylene glycol fractionation procedure.

Third, the immunoreactivity and yields of VT IgY were analyzed by analytical immunochemical methods (e.g., enzyme immunoassay (EIA) and Western blotting). EIA and Western blot analysis showed that the resulting egg preparations contained high titer IgY that reacted with both the immunizing and the heterologous toxins (i.e., rVT1 IgY reacted against both rVT1 and rVT2, and vice versa).

Fourth, VT neutralization potency was analyzed *in vitro* using a Vero cytotoxicity assay. Vero cytotoxicity of rVT1 and rVT2 could be completely inhibited by VT IgY. These antibodies also demonstrated substantial verotoxin cross-neutralization.

Fifth, the efficacy of passively administered avian verotoxin antibodies in preventing the lethal effects of verotoxin poisoning was assessed in a mouse disease model. Toxin neutralizing antibodies were administered by parenteral dosing regimens to assess the most

effective strategy for therapeutic intervention. Efficacy of verotoxin antibodies was demonstrated using multiple murine disease models. In these experiments, antibodies prevented both the morbidity and lethality of homologous and heterologous toxins using a toxin/antitoxin premix format: mice infected orally with a lethal dose of viable *E. coli* O157:H7 were protected from both morbidity and lethality when treated parenterally four hours post-infection with either rVT1 or rVT2 antibodies: and mice given a lethal dose of *E. coli* O91:H21 (a particularly virulent strain which only produces VT2c, a VT2 structural variant) and treated parenterally *up to 10 hours later* with rVT1 IgY administered parenterally were protected from both morbidity and lethality.

10

EXAMPLE 1

TOXIN ANALYSIS AND IMMUNIZATION

Purified recombinant *E. coli* O157:H7 verotoxins, rVT1 and rVT2, were obtained from Denka Sieken Co., Ltd. (Tokyo, Japan). Toxin genes were isolated, inserted into expression plasmids, and expressed in *E. coli*. Recombinant proteins were then purified by ammonium sulfate precipitation, ion exchange chromatography on DEAE Sephadex and hydroxyapatite, and gel filtration chromatography by the supplier. Upon receipt, toxins were analyzed to verify identity, purity and toxicity, as described below.

20 A. **Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).**

Samples of each toxin (2 µg) were heat-denatured in a buffer containing SDS and β-mercaptoethanol followed by electrophoresis on 10–20% gradient gels (Bio-Rad, Richmond, CA). Resolved polypeptide bands were visualized using the silver stain procedure of C.R. Merril, *et al.*, "Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins." *Science* 211: 1437-1438 (1981).

25 VT1 and VT2 are each composed of subunit A and multiple copies of subunit B. Subunit A is often nicked into fragments A1 and A2 which are linked by a disulfide bridge. As shown in Figure 1, when separated by SDS-PAGE in the presence of β-mercaptoethanol, rVT1 resolved into 3 bands that corresponded to subunit A (~31 Kda), fragment A1 (~27 Kda) and a mixture of subunit B and fragment A2 (~4 Kda). Similarly, rVT2 resolved into subunit A (~33 Kda), fragment A1 (~27 Kda) and a mixture of subunit B and fragment A2 (~8 Kda) (Figure 1). In this Figure, rVT1 is in Lane 1, and rVT2 is in Lane 2; the positions of

molecular weight markers (Kda) are shown at the left. VT component polypeptides are identified at the right.

These results are consistent with previous reports of VT1 and VT2 purified from naturally occurring toxigenic strains (V. V. Padhye *et al.*, "Purification and Physicochemical Properties of a Unique Vero Cell Cytotoxin From *Escherichia coli* O157:H7." *Biochem. Biophys. Res. Commun.* 139: 424-430 [1986]; and F. B. Kittel *et al.*, "Characterization and inactivation of verotoxin 1 produced by *Escherichia coli* O157:H7." *J. Agr. Food Chem.* 39: 141-145 [1991]).

10 B. High Performance Liquid Chromatography (HPLC).

Chromatography was performed at room temperature (RT) under isocratic conditions using a Waters 510 HPLC pump. Eluted protein was measured using a Waters 490E programmable multi-wavelength detector (Millipore Corp., Milford, MA). The VT's were separated on an 8 x 300 mm (ID) Shodex KW803 column, using 10 mM sodium phosphate, 0.15 M NaCl, pH 7.4 (phosphate buffered saline [PBS]) as the mobile phase at a flow rate of 1 ml/min.

15 The purity of non-denatured rVT's was assessed by HPLC. As shown in the chromatographs in Figure 2, each toxin eluted at approximately 10 min. as a single absorbance peak at 280 nm. By integration of the area under each peak, the rVT's were shown to be >99% pure.

20 C. Vero Cell Cytotoxicity Assay.

Cytotoxic activity of rVT1 and rVT2 was assessed using modified procedures of Padhye, *et al.* (V. V. Padhye *et al.*, "Purification and Physicochemical Properties of a Unique Vero Cell Cytotoxin From *Escherichia coli* O157:H7." *Biochem. Biophys. Res. Commun.* 139: 424-430 [1986]), and McGee, *et al.*, (Z. A. McGee, *et al.*, "Local induction of tumor necrosis factor as molecular mechanism of mucosal damage by gonococci." *Microbial Pathogenesis* 12: 333-341 [1992]). Microtiter plates (96 well, Falcon, Microtest III) were inoculated with approximately 1×10^4 Vero cells (ATCC, CCL81) per well (100 μ l) and incubated overnight at 37°C in the presence of 5% CO₂ to form Vero cell monolayers. rVT1 and rVT2 solutions were serially diluted in Medium 199 supplemented with 5% fetal bovine serum (Life Technologies, Grand Island, NY), added to each well of the microtiter plates and incubated at 37°C for 18-24 hrs. Adherent (viable) cells were stained with 0.2% crystal

violet (Mallinckrodt) in 2% ethanol. Excess stain was rinsed away and the stained cells were solubilized by adding 100 μ l of 1% SDS to each well. Absorbance of each well was measured at 570 nm, and the percent cytotoxicity of each test sample was calculated using the following formula:

5

$$\% \text{ Vero Cytotoxicity} = [1 - (\text{Absorbance Sample}/\text{Absorbance Control})] \times 100$$

To determine whether the rVT's possessed potency equivalent to published cytotoxicity values, a Vero cell cytotoxicity assay was performed (Figure 3). Between 0.01–10,000 pg of either rVT1 or rVT2 was added to Vero cells. The amounts of rVT causing 50% cell death (CD₅₀), as calculated by second degree polynomial curve fitting were 0.97 pg and 1.5 pg, for rVT1 and rVT2, respectively. These results are consistent with CD₅₀ values reported previously for naturally occurring VT1 and VT2 in the range 1–35 pg and 1–25 pg, respectively (M. Petric *et al.*, "Purification and biological properties of *Escherichia coli* verocytotoxin." FEMS Microbiol. Lett., 41: 63-68 [1987]; V. L. Tesh, *et al.*, "Comparison of relative toxicities of Shiga-Like toxins Type I and Type II for mice." Infect. Immun., 61: 3392-3402 [1993]; N. Dickie *et al.*, "Purification of an *Escherichia coli* Serogroup O157:H7 verotoxin and its detection in North American hemorrhagic colitis isolates." J. Clin. Microbiol., 27: 1973-1978 [1989]; and U. Kongmuang, *et al.*, "A simple method for 15 purification of Shiga or Shiga-Like toxin from *Shigella dysenteriae* and *Escherichia coli* O157:H7 by immunoaffinity chromatography." FEMS Microbiol. Lett., 48: 379-383 [1987]). It has been observed that toxicity is lost with storage, explaining why higher amounts of toxin 20 were used in the neutralization assays described below.

25 **D. Mouse Lethal Dose Determination.**

To verify rVT1 and rVT2 toxicity, male (20–22 g) CD-1 mice were injected intraperitoneally with varying amounts of rVT1 or rVT2 in 200 μ L phosphate buffer. Doses were selected based on published LD₅₀ values for VT1 and VT2 in CD-1 mice. To minimize the sacrifice of live animals, a full statistical toxin LD₅₀ was not determined. Mice were 30 observed for morbidity and mortality over 7-day period.

Further confirmation of rVT toxicity was obtained from mouse lethality experiments (Table 2). Mice were injected intraperitoneally with varying amounts of either rVT1 or rVT2 and observed 7 days for mortality. Within 72–120 hrs. post-injection, all of the mice died

from 100 ng of rVT1 or 10 ng of rVT2, respectively. This lethality study served as a verification of expected toxicity but not as a statistical determination of LD₅₀. Nonetheless, these results were consistent with toxicity studies which reported LD₅₀ values in CD-1 mice of 0.4–2.0 µg for purified VT1 and 0.001–1.0 µg for purified VT2 (V. L. Tesh. *et al.*,

5 "Comparison of relative toxicities of Shiga-Like toxins Type I and Type II for mice." *Infect. Immun.* 61: 3392-3402 [1993]; and A. D. O'Brien. and G. D. LaVeck. "Purification and characterization of *Shigella dysenteriae* 1-like toxin produced by *Escherichia coli*." *Infect. Immun.* 40: 675-683 [1983]).

10

Table 2.
Lethality of rVT1 in CD-1 Mice

15

20

ng VT1 Injected	Survivors/Total	Hours Post-Injection
100	7/7	24 ± 2
	5/7	48 ± 2
	0/7	72 ± 2
10	7/7	24 ± 2
	7/7	48 ± 2
	7/7	72 ± 2
1.0	6/6	24 ± 2
	6/6	48 ± 2
	6/6	72 ± 2

Table 3.
Lethality of rVT2 in CD-1 Mice

ng VT2 Injected	Survivors/Total	Hours Post-Injection
5	3/6	48 ± 2
	2/6	72 ± 2
	0/6	120 ± 2
	5/6	48 ± 2
10	4/6	72 ± 2
	0/6	120 ± 2
	6/6	48 ± 2
	6/6	72 ± 2
	6/6	120 ± 2

15 The recombinant toxins used in these studies thus appeared to contain protein components and toxicities consistent with literature reports for native toxins. Based on these structural and functional analyses, the rVT's were considered suitable as antigens to generate specific avian antibodies.

20 **E. Antigen Preparation.**

Lyophilized samples, rVT1 and rVT2 were received and each was reconstituted with 2.5 mL of deionized water to a final concentration of 100 µg/ml in phosphate buffer. To form a toxoid, the solutions were then treated with 0.4% glutaraldehyde (Mallinckrodt) at 4°C overnight and stored at -20°C thereafter. When needed, toxoid was thawed and mixed 25 5:1 (volume:volume) with GERBU adjuvant (C. C. Biotech Corporation, Poway, CA). White Leghorn laying hens were injected subcutaneously with 25 µg of either rVT1 or rVT2 toxoid in adjuvant at 2-3 week intervals.

EXAMPLE 2

30 **PEG EXTRACTION OF EGG YOLK ANTIBODY**

Hyperimmune eggs were collected after 3 immunizations with toxoid. Egg yolks were separated from whites, pooled according to their immunogen group and blended with 4 volumes of 10 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS). Polyethylene glycol

8000 (PEG) (Amresco, Solon, OH) was then added to a final concentration of 3.5% and the mixture centrifuged at 10,000 x g for 10 min. to remove the precipitated lipid fraction. IgY-rich supernatant was filtered through cheesecloth and PEG was again added to a final concentration of 12%. The solution was centrifuged as above and the resulting supernatant 5 discarded. The IgY pellet was then dissolved in PBS to either the original (1X PEG IgY) or $\frac{1}{4}$ of the original (4X PEG IgY) yolk volume, filtered through a 0.45 μ membrane and stored at 4°C.

EXAMPLE 3

ANTITOXIN IMMUNOASSAYS

A. Enzyme Immunoassay (EIA).

EIA was used to monitor antibody responses during the immunization course. Wells of 96-well Pro-Bind microtiter plates (Falcon, through Scientific Products, McGaw Park, IL) 10 were each coated with 1 μ g of rVT's (not toxoid) in PBS overnight at 2-8°C. Wells were washed 3 times with PBS containing 0.05% Tween-20 (PBS-T) to remove unbound antigen, and the remaining protein binding sites were blocked with PBS containing 1 mg/ml BSA for 15 60 min. at room temperature (RT). IgY, diluted in PBS, was then added to the wells and incubated for 1 hr. at 37°C. Wells were washed as before to remove unbound primary antibody and incubated for 1 hr. at 37°C with alkaline phosphatase-conjugated rabbit-anti-chicken IgG (Sigma Chemical Company, St. Louis, MO) diluted 1:1000 in PBS-T. Wells 20 were again washed and 1 mg/ml *p*-nitrophenyl phosphate (Sigma Chemical Company, St. Louis, MO) in 50 mM Na₂CO₃, 10 mM MgCl₂, pH 9.5 was added and allowed to incubate at 25 RT. Phosphatase activity was detected by absorbance at 410 nm using a Dynatech MR700 microtiter plate reader.

Laying Leghorn hens were immunized as described above (Example 1, part E), using glutaraldehyde-treated rVT's. Following several immunizations, eggs were collected and IgY harvested by PEG fractionation. Figures 4 and 5 show rVT1 or rVT2 specific antibody 30 responses detected using EIA at dilutions of the original yolk IgY concentration of 1:30,000 and 1:6,000, respectively. IgY fractionated similarly from unimmunized hens (*i.e.*, preimmune antibody) did not react with either antigen at test dilutions above 1:50. Although these EIA results indicate significant antibody responses, prior experience with other toxin antigens has shown that optimization of immunization regimens, including increasing the amount of

antigen, can yield titers in excess of 1:100,000 (B. S. Thalley, *et al.*, "Development of an Avian Antitoxin to Type A Botulinum Neurotoxin." in Botulinum and Tetanus Neurotoxins: Neurotransmission and Biomedical Aspects, B. R. DasGupta, (ed.) [Plenum Press, New York, 1993] pp. 467-472). As may be expected due to their structural homology and consistent with previous reports (e.g., V. V. Padhye *et al.*, "Production and characterization of monoclonal antibodies to verotoxins 1 and 2 from *Escherichia coli* O157:H7." *J. Agr. Food Chem.*, 39: 141-145 [1989]; S. C. Head *et al.*, "Purification and characterization of verocytotoxin 2." *FEMS Microbiol. Lett.*, 51: 211-216 [1988]; and N. C. Strockbine *et al.*, "Characterization of Monoclonal Antibodies against Shiga-Like Toxin from *Escherichia coli*." *Infect. Immun.*, 50: 695-700 [1985]). Figures 4 and 5 also demonstrate that antibodies generated against one toxin cross-reacted *in vitro* with the other toxin.

B. Western Blot Analysis.

Western blots (Figure 6) performed to determine the reactivity of rVT antibodies against constituent VT polypeptides showed that rVT1 and rVT2 antibodies reacted with subunit A and fragment A1 of either toxin, and with subunit B and fragment A2 of rVT1 only. In this Figure, Panel A contains preimmune IgY, Panel B contains rVT1 IgY, and Panel C contains rVT2 IgY. Lane 1 in each panel contains rVT1 (2 μ g) and Lane 2 contains rVT2 (2 μ g). Preimmune IgY was largely nonreactive to either rVT. Both rVT IgY preparations, however, failed to react with subunit B and fragment A2 of rVT2. Some explanations for this lack of measurable reactivity might include poor immunogenicity, denaturation of the immunogen during glutaraldehyde treatment, loss of conformational epitopes due to detergent or reducing agent, or poor transfer to nitrocellulose.

To resolve the high and low molecular weight components, 2 μ g each of rVT1 and rVT2 were separated by SDS-PAGE (described above) and then transferred to nitrocellulose paper using the Milliblot-SDE system (Millipore, Medford, MA) according to the manufacturer's instructions. Paper strips were stained temporarily with Ponceau S (Sigma Chemical Company, St. Louis, MO) to visualize the polypeptides and then blocked overnight in PBS containing 5% dry milk. Each strip was agitated gently in IgY diluted in PBS-T for 2 hrs. at RT. Strips were each washed with three changes of PBS-T to remove unbound primary antibody and incubated for 2 hrs. at RT with goat anti-chicken alkaline phosphatase (Kirkegaard and Perry, Gaithersburg, MD) diluted 1:500 in PBS-T containing 1 mg/ml BSA. The blots were washed as before and rinsed in 50 mM Na₂CO₃, pH 9.5. Strips were

submerged in alkaline-phosphatase substrate (5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (Kirkegaard and Perry) until sufficient signal was observed. Color development was stopped by flooding the blots with water.

5

EXAMPLE 4

IN VITRO TOXIN NEUTRALIZATION: VERO CELL ASSAY

IgY neutralization of rVT1 and rVT2 was assessed using the modified Vero 10 cytotoxicity assay described above (Example 1, part C). Various concentrations of IgY, diluted in Medium 199 supplemented with 5% fetal bovine serum (GIBCO), were mixed with sufficient toxin to cause 50% cell death and allowed to incubate at 37°C for 60 minutes. These toxin/antibody mixtures were then added to Vero cell-coated microtiter plate wells according to the procedure described above (Example 1, part C).

15 The toxin neutralization capacity of the rVT antibodies was analyzed first using a Vero cell toxicity assay. The results in Figure 7 show that rVT1 IgY neutralized completely the cytotoxic activity of rVT1 at an endpoint dilution of 1/320. Furthermore, rVT2 IgY neutralized the heterologous rVT1 toxin, but at a higher endpoint concentration.

In a similar experiment (see Figure 8), rVT1 and rVT2 antibodies were each able to 20 neutralize rVT2 at equivalent endpoint dilutions. This strong cross-neutralization correlates with the observed strong cross-reactivity of VT1 IgY with VT2 A seen on Western blots (Figure 6). These results show that IgY antibodies are able to neutralize effectively VT cytotoxicity and that the antibodies can cross-neutralize structurally-related heterologous toxins.

25

EXAMPLE 5

TOXIN NEUTRALIZATION: MOUSE ASSAYS

A. Toxin Challenge Model.

IgY in PBS was premixed with a lethal dose of toxin (as determined above) and 30 injected intraperitoneally into male CD-1 (20-22 gm) mice. Mice were observed for a 7-day period for signs of intoxication such as ruffled fur, huddling and disinclination to move, followed by hind leg paralysis, rapid breathing and death. Untreated, infected mice usually died within 12 hrs. after signs of severe illness (i.e., within 48-72 hrs. post-injection).

Once it was demonstrated that rVT antibodies were able to neutralize rVT cytotoxicity *in vitro*, protection experiments were next performed in mice. First, animals were challenged with rVT premixed with rVT IgY to determine whether toxin lethality could be neutralized under conditions optimal for antigen/antibody reaction. Tables 4 and 5 show that antibodies premixed with the homologous toxin (e.g., rVT1 with rVT1 IgY) prevented lethality of rVT. Preimmune IgY was unable to neutralize either toxin in these studies.

Table 4
Neutralization of rVT1 Using rVT IgY

100 ng rVT2 Premixed*	Survivors/Total	<i>p</i>
Preimmune Antibody	0/12	
rVT1 Antibody	12/12	< 0.001
rVT2 Antibody	12/12	< 0.001

15 *Toxin was pre-mixed with IgY and incubated for 1 hour at room temperature prior to administration.

Table 5
Neutralization of rVT2 Using rVT IgY

10 ng rVT1 Premixed*	Survivors/Total	<i>p</i>
Preimmune Antibody	0/12	
rVT1 Antibody	12/12	< 0.001
rVT2 Antibody	12/12	< 0.001

25 *Toxin was pre-mixed with IgY and incubated for 1 hour at room temperature prior to administration.

Antibodies premixed with the heterologous toxin (e.g., rVT2 with rVT1 IgY) also prevented lethality *in vivo*. These data are in contrast to previous observations where rabbit 30 polyclonal antibodies generated against either toxin were cross-reactive with the heterologous toxin by EIA and Western blot, but were unable to neutralize the heterologous toxin in either Vero cell cytotoxicity and mouse lethality assays (S. C. Head, *et al.*, "Serological differences between verocytotoxin 2 and Shiga-like toxin II," *Lancet* ii: 751 [1988]; S. C. Head *et al.*, "Purification and characterization of verocytotoxin 2," *FEMS Microbiol. Lett.*, 51: 211-216

[1988]; N. C. Strockbine *et al.*, "Characterization of Monoclonal Antibodies against Shiga-Like Toxin from *Escherichia coli*," Infect. Immun., 50: 695-700 [1985]; and V. V. Padhye *et al.*, "Purification and Physicochemical Properties of a Unique Vero Cell Cytotoxin From *Escherichia coli* O157:H7," Biochem. Biophys. Res. Commun., 139: 424-430 [1986]).

5 However, Head *et al.*, showed that VT2 B-subunit specific monoclonal antibodies neutralized VT1 weakly in a Vero cytotoxicity assay (S. C. Head, *et al.*, "Serological differences between verocytotoxin 2 and Shiga-like toxin II," Lancet ii: 751 [1988]). In a report by Donohue-Rolfe, *et al.*, a VT2 B subunit-specific monoclonal antibody neutralized both VT1 and VT2 completely in a Hela cytotoxicity assay (A. Donohue-Rolfe *et al.*, 10 "Purification of Shiga toxin and Shiga-like toxins I and II by receptor analog affinity chromatography with immobilized P1 glycoprotein and production of cross reactive monoclonal antibodies," Infect. Immun., 57: 3888-3893 [1989]).

15 These results showed for the first time complete cross-neutralization in Vero cell cytotoxicity and mouse lethality assays, revealing that VT1 and VT2 do indeed share common neutralizing epitopes. These results may indicate that hens generate different antibody specificities as compared to mammals, and/or that differences in immunization methods might have maintained the immunogenicity of conformational epitopes necessary for cross-neutralization. Nonetheless, this cross-neutralization suggests that IgY antibodies may contain 20 the range of reactivities essential for an effective antitoxin.

B. Viable organism infection model.

Streptomycin-resistant *E. coli* O157:H7 (strain 933 cu-rev) or *E. coli* O91:H21 (strain B2I 1) (both kindly provided by Dr. Alison O'Brien, Dept. of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD) were used in a murine 25 infection model described by Wadolkowski, *et al.* (E. A. Wadolkowski *et al.*, "Mouse model for colonization and disease caused by enterohemorrhagic *Escherichia coli* O157:H7," Infect. Immun., 58: 2438-2445 [1990]). Organisms were grown in Luria broth and incubated overnight at 37°C in an Environ Shaker (Lab Line, Melrose Park, IL) (T. Maniatis *et al.*, 30 Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y., [1982]). Bacterial suspensions were centrifuged at 6700 x g for 5 minutes. The resulting pellet was then washed twice with sterile PBS and resuspended in sterile 20% (w/v) sucrose. Five to 8 week-old male CD-1 mice were provided drinking water containing 5 mg/ml streptomycin sulfate *ad libitum* for 24 hrs. Food and water were then withheld for

another 16-18 hrs. after which mice were challenged orally with 10^{10} streptomycin-resistant *E. coli* O157:H7 or O91:H21. Mice were housed individually and permitted food and water containing 5 mg/ml streptomycin sulfate. IgY was injected intraperitoneally at varying times post-infection and animals observed for both morbidity and mortality for 10 days.

5 To monitor bacterial colonization in animals. 1 gram of feces was collected. homogenized, and plated onto MacConkey agar medium (Difco Laboratories. Detroit. MI) containing 100 μ g/ml streptomycin and incubated at 37°C as described by Wadolkowski, *et al.* (E. A. Wadolkowski *et al.*, "Mouse model for colonization and disease caused by enterohemorrhagic *Escherichia coli* O157:H7," Infect. Immun.. 58: 2438-2445 [1990]). The 10 serotype of *E. coli* O157:H7, 933 cu-rev excreted in feces was confirmed by slide agglutination with O- and H-specific antisera (Difco Laboratories. Detroit. MI).

15 Kidneys were removed from experimental animals and fixed in 10% buffered neutral formalin. Sections of parafilm-embedded tissue were stained with hematoxylin and eosin (General Medical Laboratories. Madison. WI) and examined by light microscopy. All tissue sections were coded to avoid bias before microscopic examination to determine renal pathology.

The toxin neutralization ability of rVT IgY was further studied using a streptomycin-treated CD-1 mouse infection model. This model was chosen because it produces definitive systemic pathology and reproducible mortality.

20 In contrast to previous studies by Wadolkowski, *et al.* (E. A. Wadolkowski *et al.*, "Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shiga-like toxin Type II," Infect. Immun.. 58: 3959-3965 [1990]), where mice were given subunit-specific monoclonal antibodies *prior* to infection, the mice in this study were inoculated orally with 2×10^{10} viable *E. coli* O157:H7 (strain 933 cu-rev) and treated 25 with rVT IgY 4 hrs. *following* inoculation. Fecal cultures showed that 10^7 - 10^8 challenge organisms per gram of feces were shed throughout the course of the experiment, thus confirming that infection was established. Tables 6 and 7 show that animals treated with either rVT1 or rVT2 IgY were protected from lethality caused by infection ($p<0.01$ and $p<0.001$, respectively) and that preimmune IgY failed to provide protection to the mice.

30

Table 6
Protection of Mice From *E. coli* O157:H7
With rVT1 IgY

IgY Treatment	Survivors/Total	p	Morbidity/Total
Preimmune Antibody	0/5		5/5
rVT1 Antibody	9/10	< 0.01	1/10

5 *IgY was administered intraperitoneally 4 hours following infection, and once daily for 10 days thereafter.

Table 7
Protection of Mice From *E. coli* O157:H7
With rVT2 IgY

IgY Treatment	Survivors/Total	p	Morbidity/Total
Preimmune Antibody	0/6		6/6
rVT2 Antibody	10/10	< 0.005	0/10

10 20 *IgY was administered intraperitoneally 4 hours following infection, and once daily for 10 days thereafter.

15 Renal histopathology (see Figure 9) of the control (preimmune IgY) animals showed dilation, degeneration and renal tubular necrosis with no glomerular damage. This is consistent with previous reports showing that renal tubular involvement occurs predominantly in this streptomycin-treated mouse infectivity model (E. A. Wadolkowski *et al.*, "Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shiga-like toxin Type II," *Infect. Immun.*, 58: 3959-3965 [1990]). Importantly, none of the survivors exhibited similar signs of morbidity though treated with IgY 4 hrs. *after* infection (see Figure 9).

25 30 Furthermore, avian antibodies generated against rVT1 were able to prevent both mortality and morbidity in a mouse model where VT2 alone is implicated in the pathogenesis and lethality of *E. coli* O157:H7 strain 933 cu-rev (E. A. Wadolkowski *et al.*, "Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shiga-like toxin Type II," *Infect. Immun.*, 58: 3959-3965 [1990]).

35 To assess the broader utility of the IgY verotoxin antibodies in treating VTEC-associated disease, the mouse infectivity study was performed using a more virulent VTEC serotype known to produce VT2c—a structural variant of VT2—but not VT1 (S. W. Lindgren

et al., "Virulence of enterohemorrhagic *Escherichia coli* O91:H21 clinical isolates in an orally infected mouse model." *Infect. Immun.* 61: 3832-3842 [1993]).

Mice were inoculated orally with 5×10^9 *E. coli* O91:H21 (strain B2F1) and treated subsequently with IgY. Notably, the heterologous rVT1 IgY protected strongly against the 5 lethal effects of the VT2c structural variant, even when administered as long as 10 hrs. following infection (Table 8). Ten hours was the longest treatment window tested in this study. Only 1 of the 8 animals treated with rVT1 IgY died ($p < 0.02$), and those that survived showed no overt signs of renal histopathology (*i.e.*, acute bilateral tubular necrosis). It can thus be concluded that rVT1 IgY completely neutralized toxicity of VT2c, indicating its 10 potential as a therapeutic for at least one other pathogenic VTEC.

Table 8
Protection of Mice From *E. coli* O91:H21
With rVT1 IgY

IgY Treatment	Survivors/Total	p	Morbidity/Total
Preimmune Antibody	0/7		7/7
rVT1 Antibody	7/8	< 0.02	1/8

20 *IgY was administered intraperitoneally 10 hours following infection, and once daily for 8 days thereafter.

These Examples highlight several important findings supporting the feasibility of using 25 verotoxin antitoxin. First, polyclonal IgY generated against either VT1 or VT2 from *E. coli* O157:H7, cross-reacted with and fully cross-neutralized the toxicity of the non-immunizing toxin both *in vitro* and *in vivo*. Second, recombinant toxins fully neutralized the toxicity of naturally-occurring toxins produced by *E. coli* O157:H7 during the course of infection. Third, 30 antibodies generated against rVT1 from *E. coli* O157:H7 could prevent morbidity and mortality in mice infected orally with lethal doses of *E. coli* O91:H21, a particularly virulent strain which only produces VT2c, suggesting their utility in preventing systemic sequelae. Because VT1 is identical to Shiga-toxin (A. D. O'Brien *et al.*, "Shiga and Shiga-like toxins. Microbial Rev.", 51: 206-220 [1987]), VT antibodies may also be useful in preventing complications stemming from *Shigella dysenteriae* infection. Finally, animals treated with VT

IgY were protected against both death and kidney damage when treated as long as 10 hrs. after infection, supporting the hypothesis that a window for antitoxin intervention exists.

These studies strongly support the use of parenterally-administered, toxin-specific IgY as a antitoxin to prevent life-threatening complications associated with *E. coli* O157:H7 and other VTEC infections. It is contemplated that this approach would be most useful in preventing HUS and other complications when administered after the onset of bloody diarrhea and before the presentation of systemic disease.

The VT IgY developed in these studies were shown to react with and neutralize both recombinant and naturally-occurring VT. The antibody titers as measured by EIA are indicative of reasonable antibody production in the hen, however much higher production levels can be obtained with larger immunizing doses.

The results from these Examples clearly demonstrate the feasibility and provide the experimental basis for development of an avian antidote for *E. coli* O157:H7 verotoxins suitable for use in humans. In contrast to previous reports showing that rabbit polyclonal VT1 and VT2 antibodies cross-reacted, but did not cross-neutralize the heterologous toxin in Vero cytotoxicity or in mouse lethality studies (e.g., V. V. Padhye *et al.*, "Production and characterization of monoclonal antibodies to verotoxins 1 and 2 from *Escherichia coli* O157:H7," *J. Agr. Food Chem.*, 39: 141-145 [1989]; S. C. Head *et al.*, "Purification and characterization of verocytotoxin 2," *FEMS Microbiol. Lett.*, 51: 211-216 [1988]; and N. C. Strockbine *et al.*, "Characterization of monoclonal antibodies against Shiga-like toxin from *Escherichia coli*," *Infect. Immun.*, 50: 695-700 [1985]), these data provide the first demonstration of cross-neutralization *in vivo*. Antibodies against one toxin neutralized completely the heterologous toxin in both Vero cytotoxicity and mouse lethality assays. Both rVT1 and rVT2 antibodies also prevented morbidity (as assessed by renal histopathology) and mortality in mice infected with lethal doses of *E. coli* O157:H7 – the etiologic agent in 90% of the documented cases of hemolytic uremic syndrome (HUS) in the U.S. (P. M. Griffin and R. V. Tauxe, "The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome," *Epidemiol. Rev.*, 13: 60 [1990]). With at least two other VTEC serotypes known to cause HUS, the finding that rVT1 antibodies neutralized a VT2 variant produced by *E. coli* O91:H21 suggests that avian polyclonal antibodies may provide an effective antidote against other verotoxin-producing *E. coli*. These data also show for the first time, that antibodies may be administered *after* infection and still protect against morbidity and mortality.

EXAMPLE 6

EXPRESSION OF TOXIN GENES

The previous Examples clearly showed that avian polyclonal antibodies to recombinant toxins protected animals infected with verotoxigenic *E. coli*. This Example includes 5 expression of toxin genes (A and B subunits alone and together as whole toxins) in suitable prokaryotic expression systems to achieve high levels of VT antigen production.

The sequence of the toxin gene has been determined (see e.g., M.P. Jackson *et al.*, "Nucleotide sequence analysis and comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from *Escherichia coli* 933." 44:109 10 [1987]). The coding regions of the A and B subunits of VT-1 are listed in SEQ ID NOS:1 and 3, respectively. The corresponding amino acid sequence of the A and B subunits of the VT-1 toxin are listed in SEQ ID NOS:2 and 4, respectively. The coding regions of the A and B subunits of VT-2 are listed in SEQ ID NOS:5 and 7, respectively. The corresponding 15 amino acid sequence of the A and B subunits of the VT-2 toxin are listed in SEQ ID NOS:6 and 8, respectively. In addition, SEQ ID NOS:9 and 10 list the sequences which direct the expression of a poly-cistronic RNA capable of directing the translation of both the A and B subunits from the VT-1 and VT-2 genes, respectively.

In choosing a strategy for recombinant VT antigen production, there are three primary technical factors to consider. First, the appropriate VT antigen components representing the 20 spectrum of toxin epitopes encountered in nature must be utilized. Second, the protein antigens must be expressed at sufficient levels and purity to enable immunization and large-scale antibody purification. Third, the neutralizing epitopes must be preserved in the immunogen and immunoabsorbant. Approaches that offer the greatest promise for high level expression of periplasmically localized, native, affinity-tagged proteins were developed. 25 Figure 10 shows the fusion constructs of VT components and affinity tags.

A. Expression of affinity-tagged C-terminal constructs.

The VT1 and VT2 A and B subunits (SEQ ID NOS:1, 3, 5 and 7) are cloned into the pET-23b vector (Novagen). This vector is designed to allow expression of native proteins 30 containing C-terminal poly-His tags. The vector utilizes a strong T7 polymerase promoter to drive high level expression of target proteins. The methionine initiation codon is engineered to contain a unique *Nde*I restriction enzyme site (CATATG). The VT1 and VT2 genes are engineered to convert the signal sequence methionine codon into a *Nde*I site utilizing PCR

mutagenesis. PCR primers were designed which contain the sequence GCCAT fused to the first 20-24 bases of the genes (starting at the ATG start codon of the signal tag: SEQ ID NOS:12-19, see Table below). Upon PCR amplification, the 5' start codon of each gene is converted to an *Nde*I site, compatible with the pET-23 vector-encoded *Nde*I site, allowing 5 cloning of the amplified genes into the vector without the addition of vector-encoded amino acids.

Primers containing the C-terminal 7 codons of each gene (21 bases) fused to the sequence CTCGAGCC were synthesized, in order to add a C-terminal poly-His tag to each gene. The underlined bases are an *Xba*I site, that is compatible with the *Xba*I site of the 10 pET-23 vector. These primers precisely delete the native stop codons, and when cloned into the pET-23 vector, add a C-terminal extension of "LeuGluHisHisHisHisHis" (SEQ ID NO: 11). The following table lists the primer pairs are utilized to create PCR fragments containing the A and B subunits derived from VT-1 and VT-2 toxin genes suitable for insertion into the pET-23b vector.

15

Table 9
Primers

Toxin Gene and Subunit	N-terminal Primer	C-terminal Primer
VT-1 Subunit A	SEQ ID NO:12	SEQ ID NO:13
VT-1 Subunit B	SEQ ID NO:14	SEQ ID NO:15
20 VT-2 Subunit A	SEQ ID NO:16	SEQ ID NO:17
VT-2 Subunit B	SEQ ID NO:18	SEQ ID NO:19
VT-1 Subunits A and B	SEQ ID NO:12	SEQ ID NO:15
VT-2 Subunits A and B	SEQ ID NO:16	SEQ ID NO:19

25 Thus, utilizing PCR amplification with the above modified N- and C-terminal primers, the A and B subunits of VT1 and VT2 are expressed as proteins containing an 8 amino acid C-terminal extension bearing an poly-histidine affinity tag. The amino acid sequence of the histidine-tagged VT-1 A subunit produced by expression from the pET-23b vector is listed in SEQ ID NO:21 (the associated DNA sequence is listed in SEQ ID NO:20); the amino acid 30 sequence of the histidine-tagged VT-1 B subunit is listed in SEQ ID NO:23 (the associated

DNA sequence is listed in SEQ ID NO:22); the amino acid sequence of the histidine-tagged VT-2 A subunit is listed in SEQ ID NO:25 (the associated DNA sequence is listed in SEQ ID NO:24); the amino acid sequence of the histidine-tagged VT-2 B subunit is listed in SEQ ID NO:27 (the associated DNA sequence is listed in SEQ ID NO:26).

5 Both subunits may be expressed from a single expression constructs by utilizing SEQ ID NOS:12 and 15 to prime synthesis of the VT-1 toxin gene and SEQ ID NOS:16 and 19 to prime synthesis of the VT-2 toxin gene. The resulting PCR products are cleaved with *Nde*I and *Xba*I, as described for the cloning of the subunit genes into the pET-23b vector. Expression of the A and B subunits from such an expression vector, results in the expression 10 of a native A subunit and a his-tagged B subunit. As the A and B subunits assemble into a complex, the presence of the his-tag on only the B subunit is sufficient to allow purification of the holotoxin on metal chelate columns as described below.

15 The proofreading *Pfu* polymerase (Stratagene) is utilized for PCR amplification to reduce the error rate during amplification. Genomic DNA from an *E. coli* O157:H7 strain is utilized as template DNA. Following the PCR, the amplification products are digested with *Nde*I and *Xba*I and cloned into the pCR-Script SK cloning vehicle (Stratagene) to permit 20 DNA sequence analysis of the amplified products. The DNA sequence analysis is performed to ensure that no base changes are introduced during amplification. Once the desired clones are identified by DNA sequencing, the inserts are then excised utilizing *Nde*I and *Xba*I, and cloned into a similarly cut pET-23b vector to create the expression constructs. According to the published sequences, neither the VT1 nor VT2 genes contain either of these restriction sites.

25 The poly-His-tagged proteins produced by expression of the VT-1 and VT-2 gene sequences in the pET-23b constructs are then purified by IMAC. This method uses metal-chelate affinity chromatography to purify native or denatured proteins which have histidine tails (see e.g., K. J. Petty, "Metal-Chelate Affinity Chromatography," in Current Protocols in Molecular Biology, Supplement 24, Unit 10.11B [1993]).

B. Expression of Toxin Containing N-terminal Affinity Tags

30 Two expression systems, pMal-p2 and pFLAG-1 are utilized to attach an N-terminal affinity tag to the A subunits from the VT-1 and VT-2 toxins.

MBP-tagged constructs. To construct A chains containing the maltose binding protein (MBP) at the N-terminus of the A subunit, PCR amplified gene products are cloned into the

5 pMal-p2 vector (New England Biolabs) as C-terminal fusions to a periplasmically-secreted version of the MBP. The MBP selectively binds to amylose resins and serves as an affinity tag on the MBP/A subunit fusion protein. The pMal-p2 vector contains an engineered factor Xa cleavage site, which permits the removal of the affinity tag (*i.e.*, MBP) from the fusion protein after purification.

10 The MBP/A subunit fusions are generated as follows. The VT1 and VT2 A subunits are PCR-amplified utilizing the following DNA primers. SEQ ID NOS:28-31: SEQ ID NOS:28 and 29 comprise the 5' and 3' primers, respectively, for the amplification of the VT1 A subunit; SEQ ID NOS:30 and 31 comprise the 5' and 3' primers, respectively, for the 15 amplification of the VT2 A subunit. In both cases, the 5' or N-terminal primer contains the sequence CGGAATTC fused to the first codon of the mature polypeptide (rather than the start of the signal peptide, since the MBP signal peptide is utilized). These 5' primers contain an engineered *Eco*RI site that is not contained internally in either gene, that is compatible with the *Eco*RI site of the pMal-p2 vector. The 3' or C-terminal primers incorporate an *Xba*I site 20 as described above for the generation of the His-tagged toxins, but in this case, the 3' primer is designed to include the natural termination codon of the A subunits.

25 The genes are amplified, cloned into pCR-Script SK, and sequenced as described above. The inserts are then excised with *Eco*RI and *Xba*I, and cloned into *Eco*RI/*Sal*I-cleaved pMal-p2 vector (*Sal*I and *Xba*I sites are compatible). This construct allows expression and secretion of the VT1 and VT2 A subunit genes as C-terminal fusions with MBP. The amino acid sequence of the MBP/VT-1A fusion protein is listed in SEQ ID NO:33 (the associated DNA sequence is listed in SEQ ID NO:32). The amino acid sequence of the MBP/VT-2A fusion protein is listed in SEQ ID NO:35 (the associated DNA sequence is listed in SEQ ID NO:34).

30 The resulting fusion proteins are then affinity purified on an amylose column and the bound fusion protein is eluted under mild conditions by competition with maltose. The MBP N-terminal-tagged A subunits are cleaved with factor Xa and the MBP is removed by chromatography on an amylose column. The resulting A subunits which contain a 4 amino acid N-terminal extension are then used as immunogens.

35

Flag tag constructs. In an alternative embodiment, the VT1 and VT2 A subunit genes are engineered to contain the "flag tag" through the use of the pFLAG-1 vector system. The flag tag is located between the *OmpA* secretion signal sequence and the authentic N-

terminus of the target protein in the pFlag-1 vector. To construct N-terminal flag-tagged A chains, the *EcoRI/XbaI* A subunit PCR fragments (generated as described above for the MBP fusion proteins) are cloned into identically cleaved pFlag-1 vector (Eastman-Kodak), to produce an expression construct utilizing the *OmpA* signal peptide for secretion of A subunit fusion proteins containing the flag peptide at the N-terminus. After secretion, the periplasmic protein contains the N-terminal 8 amino acid flag tag, followed by 4 vector-encoded amino acids fused to the recombinant A subunit. The amino acid sequence of the flag tag/VT-1 A subunit fusion protein is listed in SEQ ID NO:37 (the associated DNA sequence is listed in SEQ ID NO:36). The amino acid sequence of the flag tag/VT-2 A subunit fusion protein is listed in SEQ ID NO:39 (the associated DNA sequence is listed in SEQ ID NO:38).

The flag tag fusion proteins are then purified by immunoaffinity chromatography utilizing a calcium-dependent monoclonal antibody (Antiflag M1; Eastman-Kodak). Mild elution of purified protein is achieved by chelating the calcium in the column buffer with ethylenediamine tetraacetic acid (EDTA).

15

C. Evaluation of fusion construct expression.

The fusion constructs described above are expressed in *E. coli* strain BL21, or T7 polymerase-containing derivatives [e.g., BL21(DE3), BL21(DE3) pLysS, BL21(DE3)pLysE] (Novagen) for pET plasmids, and periplasmically-secreted recombinant protein purified by affinity chromatography. Recombinant proteins are analyzed for correct conformation by testing the following parameters:

20

- a) It is believed that the B subunit must associate into pentamers to be conformationally correct. This is assessed by reducing and native SDS-PAGE analyses of native and chemically-cross-linked proteins and sizing HPLC;
- b) It is believed that a properly folded A subunit is expected to retain its native enzymatic activity. This is tested by its capacity to inhibit protein synthesis in an *in vitro* toxicity assay;
- c) It is believed that *in vitro* toxicity of assembled recombinant holotoxin is compared to commercially available holotoxins to determine whether recombinant A and B subunits can assemble into functional holotoxin. The

30

purified N-terminal-tagged A subunits (after cleavage and purification from MBP or untreated flag-tagged proteins) are combined *in vitro* with the corresponding B chains, and their toxicity evaluated utilizing a quantitative microtiter cytotoxicity assay, such as that described by M.K. Gentry and M. 5 Dalrymple, "Quantitative Microtiter Cytotoxicity Assay for *Shigella* Toxin," *J. Clin. Microbiol.*, 12:361-366 (1980).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: OPHIDIAN PHARMACEUTICALS, INC.

(ii) TITLE OF INVENTION: TREATMENT FOR VEROTOXIN-PRODUCING E. COLI

(iii) NUMBER OF SEQUENCES: 39

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: MEDLEN & CARROLL
 (B) STREET: 220 MONTGOMERY STREET, SUITE 2200
 (C) CITY: SAN FRANCISCO
 (D) STATE: CALIFORNIA
 (E) COUNTRY: UNITED STATES OF AMERICA
 (F) ZIP: 94104

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
 (B) FILING DATE:
 (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: CARROLL, PETER G.
 (B) REGISTRATION NUMBER: 32,837
 (C) REFERENCE/DOCKET NUMBER: OPHD-02171

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (415) 705-8410
 (B) TELEFAX: (415) 397-8338

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 945 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: 1..945

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATG AAA ATA ATT ATT TTT AGA GTG CTA ACT TTT TTC TTT GTT ATC TTT	48
Met Lys Ile Ile Ile Phe Arg Val Leu Thr Phe Phe Val Ile Phe	
1 5 10 15	

TCA GTT AAT GTG GTG GCG AAG GAA TTT ACC TTA GAC TTC TCG ACT GCA	96
Ser Val Asn Val Val Ala Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala	
20 25 30	

AAG ACG TAT GTA GAT TCG CTG AAT GTC ATT CGC TCT GCA ATA GGT ACT	144
Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr	
35 40 45	

CCA TTA CAG ACT ATT TCA TCA GGA GGT ACG TCT TTA CTG ATG ATT GAT	192
Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp	
50 55 60	

AGT GGC TCA GGG GAT AAT TTG TTT GCA GTT GAT GTC AGA GGG ATA GAT Ser Gly Ser Gly Asp Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp 65 70 75 80	240
GCA GAG GAA GGG CGG TTT AAT AAT CTA CGG CTT ATT GTT GAA CGA AAT Ala Glu Glu Gly Arg Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn 85 90 95	288
AAT TTA TAT GTG ACA GGA TTT GTT AAC AGG ACA AAT AAT GTT TTT TAT Asn Leu Tyr Val Thr Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr 100 105 110	336
CGC TTT GCT GAT TTT TCA CAT GTT ACC TTT CCA GGT ACA ACA GCG GTT Arg Phe Ala Asp Phe Ser His Val Thr Phe Pro Gly Thr Thr Ala Val 115 120 125	384
ACA TTG TCT GGT GAC AGT AGC TAT ACC ACG TTA CAG CGT GTT GCA GGG Thr Leu Ser Gly Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly 130 135 140	432
ATC AGT CGT ACG GGG ATG CAG ATA AAT CGC CAT TCG TTG ACT ACT TCT Ile Ser Arg Thr Gly Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser 145 150 155 160	480
TAT CTG GAT TTA ATG TCG CAT AGT GGA ACC TCA CTG ACG CAG TCT GTG Tyr Leu Asp Leu Met Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val 165 170 175	528
GCA AGA GCG ATG TTA CGG TTT GTT ACT GTG ACA GCT GAA GCT TTA CGT Ala Arg Ala Met Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg 180 185 190	576
TTT CGG CAA ATA CAG AGG GGA TTT CGT ACA ACA CTG GAT GAT CTC AGT Phe Arg Gln Ile Gln Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser 195 200 205	624
GGG CGT TCT TAT GTA ATG ACT GCT GAA GAT GTT GAT CTT ACA TTG AAC Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn 210 215 220	672
TGG GGA AGG TTG AGT AGC GTC CTG CCT GAC TAT CAT GGA CAA GAC TCT Trp Gly Arg Leu Ser Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser 225 230 235 240	720
GTT CGT GTA GGA AGA ATT TCT TTT GGA AGC ATT AAT GCA ATT CTG GGA Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly 245 250 255	768
AGC GTG GCA TTA ATA CTG AAT TGT CAT CAT CAT GCA TCG CGA GTT GCC Ser Val Ala Leu Ile Leu Asn Cys His His Ala Ser Arg Val Ala 260 265 270	816
AGA ATG GCA TCT GAT GAG TTT CCT TCT ATG TGT CCG GCA GAT GGA AGA Arg Met Ala Ser Asp Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg 275 280 285	864
GTC CGT GGG ATT ACG CAC AAT AAA ATA TTG TGG GAT TCA TCC ACT CTG Val Arg Gly Ile Thr His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu 290 295 300	912
GGG GCA ATT CTG ATG CGC AGA ACT ATT AGC AGT Gly Ala Ile Leu Met Arg Arg Thr Ile Ser Ser 305 310 315	945

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 315 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Lys Ile Ile Ile Phe Arg Val Leu Thr Phe Phe Phe Val Ile Phe
 1 5 10 15

Ser Val Asn Val Val Ala Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala
 20 25 30

Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr
 35 40 45

Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp
 50 55 60

Ser Gly Ser Gly Asp Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp
 65 70 75 80

Ala Glu Glu Gly Arg Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn
 85 90 95

Asn Leu Tyr Val Thr Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr
 100 105 110

Arg Phe Ala Asp Phe Ser His Val Thr Phe Pro Gly Thr Thr Ala Val
 115 120 125

Thr Leu Ser Gly Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly
 130 135 140

Ile Ser Arg Thr Gly Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser
 145 150 155 160

Tyr Leu Asp Leu Met Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val
 165 170 175

Ala Arg Ala Met Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg
 180 185 190

Phe Arg Gln Ile Gln Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser
 195 200 205

Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn
 210 215 220

Trp Gly Arg Leu Ser Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser
 225 230 235 240

Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly
 245 250 255

Ser Val Ala Leu Ile Leu Asn Cys His His His Ala Ser Arg Val Ala
 260 265 270

Arg Met Ala Ser Asp Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg
 275 280 285

Val Arg Gly Ile Thr His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu
 290 295 300

Gly Ala Ile Leu Met Arg Arg Thr Ile Ser Ser
 305 310 315

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 267 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
- ii) MOLECULE TYPE: DNA (genomic)
- ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..267

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATG AAA AAA ACA TTA TTA ATA GCT GCA TCG CTT TCA TTT TTT TCA GCA Met Lys Lys Thr Leu Leu Ile Ala Ala Ser Leu Ser Phe Phe Ser Ala	48
1 5 10 15	
AGT GCG CTG GCG ACG CCT GAT TGT GTA ACT GGA AAG GTG GAG TAT ACA Ser Ala Leu Ala Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr	96
20 25 30	
AAA TAT AAT GAT GAC GAT ACC TTT ACA GTT AAA GTG GGT GAT AAA GAA Lys Tyr Asn Asp Asp Asp Thr Phe Thr Val Lys Val Gly Asp Lys Glu	144
35 40 45	
TTA TTT ACC AAC AGA TGG AAT CTT CAG TCT CTT CTT CTC AGT GCG CAA Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu Leu Ser Ala Gln	192
50 55 60	
ATT ACG GGG ATG ACT GTA ACC ATT AAA ACT AAT GCC TGT CAT AAT GGA Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly	240
65 70 75 80	
GGG GGA TTC AGC GAA GTT ATT TTT CGT Gly Gly Phe Ser Glu Val Ile Phe Arg	267
85	

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 89 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Met Lys Lys Thr Leu Leu Ile Ala Ala Ser Leu Ser Phe Phe Ser Ala
1 5 10 15

Ser Ala Leu Ala Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr
20 25 30

Lys Tyr Asn Asp Asp Asp Thr Phe Thr Val Lys Val Gly Asp Lys Glu
35 40 45

Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu Leu Ser Ala Gln
50 55 60

Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly
65 70 75 80

Gly Gly Phe Ser Glu Val Ile Phe Arg
85

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 954 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 1..954

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATG AAG TGT ATA TTA TTT AAA TGG GTA CTG TGC CTG TTA CTG GGT TTT	48
Met Lys Cys Ile Leu Phe Lys Trp Val Leu Cys Leu Leu Leu Gly Phe	
1 5 10 15	
TCT TCG GTA TCC TAT TCC CCG GAG TTT ACG ATA GAC TTT TCG ACC CAA	96
Ser Ser Val Ser Tyr Ser Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln	
20 25 30	
CAA AGT TAT GTC TCT TCG TTA AAT AGT ATA CGG ACA GAG ATA TCG ACC	144
Gln Ser Tyr Val Ser Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr	
35 40 45	
CCT CTT GAA CAT ATA TCT CAG GGG ACC ACA TCG GTG TCT GTT ATT AAC	192
Pro Leu Glu His Ile Ser Gln Gly Thr Ser Val Ser Val Ile Asn	
50 55 60	
CAC ACC CAC GGC AGT TAT TTT GCT GTG GAT ATA CGA GGG CTT GAT GTC	240
His Thr His Gly Ser Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val	
65 70 75 80	
TAT CAG GCG CGT TTT GAC CAT CTT CGT CTG ATT ATT GAG CAA AAT AAT	288
Tyr Gln Ala Arg Phe Asp His Leu Arg Leu Ile Glu Gln Asn Asn	
85 90 95	
TTA TAT GTG GCA GGG TTC GTT AAT ACG GCA ACA AAT ACT TTC TAC CGT	336
Leu Tyr Val Ala Gly Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg	
100 105 110	
TTT TCA GAT TTT ACA CAT ATA TCA GTG CCC GGT GTG ACA ACG GTT TCC	384
Phe Ser Asp Phe Thr His Ile Ser Val Pro Gly Val Thr Thr Val Ser	
115 120 125	
ATG ACA ACG GAC AGC AGT TAT ACC ACT CTG CAA CGT GTC GCA GCG CTG	432
Met Thr Thr Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu	
130 135 140	
GAA CGT TCC GGA ATG CAA ATC AGT CGT CAC TCA CTG GTT TCA TCA TAT	480
Glu Arg Ser Gly Met Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr	
145 150 155 160	
CTG GCG TTA ATG GAG TTC AGT GGT AAT ACA ATG ACC AGA GAT GCA TCC	528
Leu Ala Leu Met Glu Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser	
165 170 175	
AGA GCA GTT CTG CGT TTT GTC ACT GTC ACA GCA GAA GCC TTA CGC TTC	576
Arg Ala Val Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe	
180 185 190	
AGG CAG ATA CAG AGA GAA TTT CGT CAG GCA CTG TCT GAA ACT GCT CCT	624
Arg Gln Ile Gln Arg Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro	
195 200 205	
GTC TAT ACG ATG ACG CCG GGA GAC GTG GAC CTC ACT CTG AAC TGG GGG	672
Val Tyr Thr Met Thr Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly	
210 215 220	

CGA ATC AGC AAT GTG CTT CCG GAG TAT CGG GGA GAG GAT GGT GTC AGA	720
Arg Ile Ser Asn Val Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg	
225 230 235 240	
GTG GGG AGA ATA TCC TTT AAT AAT ATA TCA GCG ATA CTG GGG ACT GTG	768
Val Gly Arg Ile Ser Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val	
245 250 255	
GCC GTT ATA CTG AAT TGC CAT CAT CAG GGG GCG CGT TCT GTT CGC GCC	816
Ala Val Ile Leu Asn Cys His His Gln Gly Ala Arg Ser Val Arg Ala	
260 265 270	
GTG AAT GAA GAG AGT CAA CCA GAA TGT CAG ATA ACT GGC GAC AGG CCT	864
Val Asn Glu Glu Ser Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Prc	
275 280 285	
GTT ATA AAA ATA AAC AAT ACA TTA TGG GAA AGT AAT ACA GCT GCA GCG	912
Val Ile Lys Ile Asn Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala	
290 295 300	
TTT CTG AAC AGA AAG TCA CAG TTT TTA TAT ACA ACG GGT AAA	954
Phe Leu Asn Arg Lys Ser Gln Phe Leu Tyr Thr Thr Gly Lys	
305 310 315	

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 318 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Lys Cys Ile Leu Phe Lys Trp Val Leu Cys Leu Leu Leu Gly Phe	
1 5 10 15	
Ser Ser Val Ser Tyr Ser Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln	
20 25 30	
Gln Ser Tyr Val Ser Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr	
35 40 45	
Pro Leu Glu His Ile Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn	
50 55 60	
His Thr His Gly Ser Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val	
65 70 75 80	
Tyr Gln Ala Arg Phe Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn	
85 90 95	
Leu Tyr Val Ala Gly Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg	
100 105 110	
Phe Ser Asp Phe Thr His Ile Ser Val Pro Gly Val Thr Thr Val Ser	
115 120 125	
Met Thr Thr Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu	
130 135 140	
Glu Arg Ser Gly Met Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr	
145 150 155 160	
Leu Ala Leu Met Glu Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser	
165 170 175	
Arg Ala Val Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe	
180 185 190	

Arg Gln Ile Gln Arg Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro
 195 200 205
 Val Tyr Thr Met Thr Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly
 210 215 220
 Arg Ile Ser Asn Val Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg
 225 230 235 240
 Val Gly Arg Ile Ser Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val
 245 250 255
 Ala Val Ile Leu Asn Cys His His Gln Gly Ala Arg Ser Val Arg Ala
 260 265 270
 Val Asn Glu Glu Ser Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro
 275 280 285
 Val Ile Lys Ile Asn Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala
 290 295 300
 Phe Leu Asn Arg Lys Ser Gln Phe Leu Tyr Thr Gly Lys
 305 310 315

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 267 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..267
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATG AAG AAG ATG TTT ATG GCG GTT TTA TTT GCA TTA GCT TCT GTT AAT	48
Met Lys Lys Met Phe Met Ala Val Leu Phe Ala Leu Ala Ser Val Asn	
1 5 10 15	
GCA ATG GCG GCG GAT TGT GCT AAA GGT AAA ATT GAG TTT TCC AAG TAT	96
Ala Met Ala Ala Asp Cys Ala Lys Gly Lys Ile Glu Phe Ser Lys Tyr	
20 25 30	
AAT GAG GAT GAC ACA TTT ACA GTG AAG GTT GAC GGG AAA GAA TAC TGG	144
Asn Glu Asp Asp Thr Phe Thr Val Lys Val Asp Gly Lys Glu Tyr Trp	
35 40 45	
ACC AGT CGC TGG AAT CTG CAA CCG TTA CTG CAA AGT GCT CAG TTG ACA	192
Thr Ser Arg Trp Asn Leu Gln Pro Leu Leu Gln Ser Ala Gln Leu Thr	
50 55 60	
GGA ATG ACT GTC ACA ATC AAA TCC AGT ACC TGT GAA TCA GGC TCC GGA	240
Gly Met Thr Val Thr Ile Lys Ser Ser Thr Cys Glu Ser Gly Ser Gly	
65 70 75 80	
TTT GCT GAA GTG CAG TTT AAT AAT GAC	267
Phe Ala Glu Val Gln Phe Asn Asn Asp	
85	

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 89 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Lys Lys Met Phe Met Ala Val Leu Phe Ala Leu Ala Ser Val Asn
 1 5 10 15

Ala Met Ala Ala Asp Cys Ala Lys Gly Lys Ile Glu Phe Ser Lys Tyr
 20 25 30

Asn Glu Asp Asp Thr Phe Thr Val Lys Val Asp Gly Lys Glu Tyr Trp
 35 40 45

Thr Ser Arg Trp Asn Leu Gln Pro Leu Leu Gln Ser Ala Gln Leu Thr
 50 55 60

Gly Met Thr Val Thr Ile Lys Ser Ser Thr Cys Glu Ser Gly Ser Gly
 65 70 75 80

Phe Ala Glu Val Gln Phe Asn Asn Asp
 85

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1241 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATGAAAATAA TTATTTTAG AGTGCTAACT TTTTCTTTG TTATCTTTG AGTTAATGTG	60
GTGGCGAAGG AATTACCTT AGACTTCTCG ACTGCAAAGA CGTATGTAGA TTGCTGAAT	120
GTCATTCGCT CTGCAATAGG TACTCCATTA CAGACTATT CATCAGGAGG TACGTCTTA	180
CTGATGATTG ATAGTGGCTC AGGGATAAT TTGTTGCAG TTGATGTCAG AGGGATAGAT	240
GCAGAGGAAG GGCGGTTAA TAATCTACGG CTTATTGTT AACGAAATAA TTTATATGTG	300
ACAGGATTG TTAACAGGAC AAATAATGTT TTTTATCGCT TTGCTGATT TTCACATGTT	360
ACCTTCCAG GTACAACAGC GGTTACATTG TCTGGTGACA GTAGCTATAC CACGTTACAG	420
CGTGTGCAAG GGATCAGTCG TACGGGGATG CAGATAAACG GCCATTGTT GACTACTCT	480
TATCTGGATT TAATGTCGCA TAGTGGAACC TCACTGACGC AGTCTGTGGC AAGAGCGATG	540
TTACGGTTG TTACTGTGAC AGCTGAAGCT TTACGTTTC GGCAAATACA GAGGGGATTT	600
CGTACAACAC TGGATGATCT CAGTGGCGT TCTTATGTAA TGACTGCTGA AGATGTTGAT	660
CTTACATTGA ACTGGGGAAAG GTTGAGTAGC GTCCTGCCTG ACTATCATGG ACAAGACTCT	720
GTTCGTGTAG GAAGAATTTC TTTTGGAAAGC ATTAATGCAA TTCTGGGAAG CGTGGCATTAA	780
ATACTGAATT GTCATCATCA TGCATCGCGA GTTGCAGAA TGGCATCTGA TGAGTTTCCT	840
TCTATGTGTC CGGCAGATGG AAGAGTCCGT GGGATTACGC ACAATAAAAT ATTGTGGAT	900

TCATCCACTC TGGGGCAAT TCTGATGCGC AGAACTATTA GCAGTTGAAAC AGGGGGTAAA	960
TAAAGGAGTT AAGCATGAAA AAAACATTAT TAATAGCTGC ATCGCTTCAG TTTTTTCAG	1020
CAAGTGCGCT GGCACGCCT GATTGTGTAAGTGGAAAGGT GGAGTATACAAATATAATG	1080
ATGACGATAC CTTTACAGTT AAAGTGGGTG ATAAAGAATT ATTTACCAAC AGATGGAATC	1140
TTCAGTCTCT TCTTCTCAGT GCGCAAATTA CGGGGATGAC TGTAACCATT AAAACTAATG	1200
CCTGTCATAA TGGAGGGGAA TTCAGCGAAG TTATTTTCG T	1241

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1235 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATGAAGTGTAA TATTATTTAA ATGGGTACTG TGCCTGTTAC TGGGTTTTTC TTGGTATCC	60
TATTCCCGGG AGTTTACGAT AGACTTTTCG ACCCAACAAA GTTATGTCTC TTGTTAAAT	120
AGTATAACGGA CAGAGATATC GACCCCTCTT GAACATATAT CTCAGGGGAC CACATCGGTG	180
TCTGTTATTA ACCACACCCA CGGCAGTTAT TTTGCTGTGG ATATACGAGG GCTGATGTC	240
TATCAGGCAGC GTTTTGACCA TCTTCGTCTG ATTATTGAGC AAAATAATT ATATGTGGCA	300
GGGTTCGTTA ATACGGCAAC AAATACTTTC TACCGTTTT CAGATTTAC ACATATATCA	360
GTGCCCGGTG TGACAACGGT TTCCATGACA ACGGACAGCA GTTATACCAC TCTGAAACGT	420
GTCGAGCGC CGGAACGTTC CGGAATGCAA ATCAGTCGTC ACTCACTGGT TTCACTCATAT	480
CTGGCGTTAA TGGAGTTCAAGTGGTAATACA ATGACCAGAG ATGCATCCAG AGCAGTTCTG	540
CGTTTTGTCA CTGTCACAGC AGAACGCTTA CGCTTCAGGC AGATACAGAG AGAATTCGT	600
CAGGCAGCTGT CTGAAACTGTC TCCTGTTAT ACGATGACGC CGGGAGACGT GGACCTCACT	660
CTGAACTGGG GGCGAACATCAG CAATGTGCTT CCGGAGTATC GGGGAGAGGA TGGTGTCAAGA	720
GTGGGGAGAA TATCCTTAA TAATATATCA GCGATACTGG GGACTGTGGC CGTTATACTG	780
AATTGCCATC ATCAGGGGGC GCGTTCTGTT CGCGCCGTGA ATGAAGAGAG TCAACCAGAA	840
TGTCAGATAA CTGGCGACAG GCCTGTTATA AAAATAACAA ATACATTATG GGAAAGTAAT	900
ACAGCTGCAG CGTTTCTGAA CAGAAAGTCA CAGTTTTAT ATACAACGGG TAAATAAAGG	960
AGTTAACGCAT GAAGAAGATG TTTATGGCGG TTTTATTGTC ATTAGCTTCT GTTAATGCAA	1020
TGGCGGCAGGA TTGTGCTAAA GGTAAGATTG AGTTTCCAA GTATAATGAG GATGACACAT	1080
TTACAGTGAA GGTTGACGGG AAAGAATACT GGACCAAGTCG CTGGAATCTG CAAACGTTAC	1140
TGCAAAAGTGC TCAGTTGACA GGAATGACTG TCACAATCAA ATCCAGTACCG TGTGAATCAG	1200
GCTCCGGATT TGCTGAAGTG CAGTTAATAA ATGAC	1235

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids

- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Leu Glu His His His His His His
1 5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCCATATGAA AATAATTATT TTTAGAGTG

29

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGCTCGAGAC TGCTAATAGT TCTGGCAT

29

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCCATATGAA AAAAACATTA TTAATAGC

28

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGCTCGAGAC GAAAAATAAC TTCGCTGAA

29

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGCATATGAA GTGTATATTA TTTAAATGG

29

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GGCTCGAGTT TACCCGTTGT ATATAAAAAC

30

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CGCATATGAA GAAGATGTTT ATGGCG

26

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGCTCGAGGT CATTATTAAC CTGCACTTC

29

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 969 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..969

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATG AAA ATA ATT ATT TTT AGA GTG CTA ACT TTT TTC TTT GTT ATC TTT

48

Met Lys Ile Ile Ile Phe Arg Val Leu Thr Phe Phe Phe Val Ile Phe		
1 5 10 15		
TCA GTT AAT GTG GTG GCG AAG GAA TTT ACC TTA GAC TTC TCG ACT GCA		96
Ser Val Asn Val Val Ala Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala		
20 25 30		
AAG ACG TAT GTA GAT TCG CTG AAT GTC ATT CGC TCT GCA ATA GGT ACT		144
Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr		
35 40 45		
CCA TTA CAG ACT ATT TCA TCA GGA GGT ACG TCT TTA CTG ATG ATT GAT		192
Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp		
50 55 60		
AGT GGC TCA GGG GAT AAT TTG TTT GCA GTT GAT GTC AGA GGG ATA GAT		240
Ser Gly Ser Gly Asp Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp		
65 70 75 80		
GCA GAG GAA GGG CGG TTT AAT AAT CTA CGG CTT ATT GTT GAA CGA AAT		288
Ala Glu Glu Gly Arg Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn		
85 90 95		
AAT TTA TAT GTG ACA GGA TTT GTT AAC AGG ACA AAT AAT GTT TTT TAT		336
Asn Leu Tyr Val Thr Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr		
100 105 110		
CGC TTT GCT GAT TTT TCA CAT GTT ACC TTT CCA GGT ACA ACA GCG GTT		384
Arg Phe Ala Asp Phe Ser His Val Thr Phe Pro Gly Thr Thr Ala Val		
115 120 125		
ACA TTG TCT GGT GAC AGT AGC TAT ACC ACG TTA CAG CGT GTT GCA GGG		432
Thr Leu Ser Gly Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly		
130 135 140		
ATC AGT CGT ACG GGG ATG CAG ATA AAT CGC CAT TCG TTG ACT ACT TCT		480
Ile Ser Arg Thr Gly Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser		
145 150 155 160		
TAT CTG GAT TTA ATG TCG CAT AGT GGA ACC TCA CTG ACG CAG TCT STG		528
Tyr Leu Asp Leu Met Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val		
165 170 175		
GCA AGA GCG ATG TTA CGG TTT GTT ACT GTG ACA GCT GAA GCT TTA CGT		576
Ala Arg Ala Met Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg		
180 185 190		
TTT CGG CAA ATA CAG AGG GGA TTT CGT ACA ACA CTG GAT GAT CTC AGT		624
Phe Arg Gln Ile Gln Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser		
195 200 205		
GGG CGT TCT TAT GTA ATG ACT GCT GAA GAT GTT GAT CTT ACA TTG AAC		672
Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn		
210 215 220		
TGG GGA AGG TTG AGT AGC GTC CTG CCT GAC TAT CAT GGA CAA GAC TCT		720
Trp Gly Arg Leu Ser Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser		
225 230 235 240		
GTT CGT GTA GGA AGA ATT TCT TTT GGA AGC ATT AAT GCA ATT CTG GGA		768
Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly		
245 250 255		
AGC GTG GCA TTA ATA CTG AAT TGT CAT CAT GCA TCG CGA GTT GCC		816
Ser Val Ala Leu Ile Leu Asn Cys His His Ala Ser Arg Val Ala		
260 265 270		
AGA ATG GCA TCT GAT GAG TTT CCT TCT ATG TGT CCG GCA GAT GGA AGA		864
Arg Met Ala Ser Asp Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg		
275 280 285		

GTC CGT GGG ATT ACG CAC AAT AAA ATA TTG TGG GAT TCA TCC ACT CTG	912
Val Arg Gly Ile Thr His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu	
290 295 300	
GGG GCA ATT CTG ATG CGC AGA ACT ATT AGC AGT CTC GAG CAC CAC CAC	960
Gly Ala Ile Leu Met Arg Arg Thr Ile Ser Ser Leu Glu His His His	
305 310 315 320	
CAC CAC CAC	969
His His His	

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 323 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Met Lys Ile Ile Ile Phe Arg Val Leu Thr Phe Phe Phe Val Ile Phe	
1 5 10 15	
Ser Val Asn Val Val Ala Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala	
20 25 30	
Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr	
35 40 45	
Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp	
50 55 60	
Ser Gly Ser Gly Asp Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp	
65 70 75 80	
Ala Glu Glu Gly Arg Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn	
85 90 95	
Asn Leu Tyr Val Thr Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr	
100 105 110	
Arg Phe Ala Asp Phe Ser His Val Thr Phe Pro Gly Thr Thr Ala Val	
115 120 125	
Thr Leu Ser Gly Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly	
130 135 140	
Ile Ser Arg Thr Gly Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser	
145 150 155 160	
Tyr Leu Asp Leu Met Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val	
165 170 175	
Ala Arg Ala Met Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg	
180 185 190	
Phe Arg Gln Ile Gln Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser	
195 200 205	
Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn	
210 215 220	
Trp Gly Arg Leu Ser Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser	
225 230 235 240	
Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly	
245 250 255	

Ser Val Ala Leu Ile Leu Asn Cys His His His Ala Ser Arg Val Ala
 260 265 270
 Arg Met Ala Ser Asp Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg
 275 280 285
 Val Arg Gly Ile Thr His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu
 290 295 300
 Gly Ala Ile Leu Met Arg Arg Thr Ile Ser Ser Leu Glu His His His
 305 310 315 320
 His His His

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 294 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..294

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATG AAA AAA ACA TTA TTA ATA GCT GCA TCG CTT TCA TTT TTT TCA GCA	48
Met Lys Lys Thr Leu Leu Ile Ala Ala Ser Leu Ser Phe Phe Ser Ala	
1 5 10 15	
AGT GCG CTG GCG ACG CCT GAT TGT GTA ACT GGA AAG GTG GAG TAT ACA	96
Ser Ala Leu Ala Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr	
20 25 30	
AAA TAT AAT GAT GAC GAT ACC TTT ACA GTT AAA GTG GGT GAT AAA GAA	144
Lys Tyr Asn Asp Asp Asp Thr Phe Thr Val Lys Val Gly Asp Lys Glu	
35 40 45	
TTA TTT ACC AAC AGA TGG AAT CTT CAG TCT CTT CTC AGT GCG CAA	192
Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu Ser Ala Gln	
50 55 60	
ATT ACG GGG ATG ACT GTA ACC ATT AAA ACT AAT GCC TGT CAT AAT GGA	240
Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly	
65 70 75 80	
GGG GGA TTC AGC GAA GTT ATT TTT CGT CTC GAG CAC CAC CAC CAC	288
Gly Gly Phe Ser Glu Val Ile Phe Arg Leu Glu His His His His His	
85 90 95	
CAC TG	294
His	

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 97 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Met Lys Lys Thr Leu Leu Ile Ala Ala Ser Leu Ser Phe Phe Ser Ala

1	5	10	15
Ser Ala Leu Ala Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr			
20	25	30	
Lys Tyr Asn Asp Asp Asp Thr Phe Thr Val Lys Val Gly Asp Lys Glu			
35	40	45	
Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu Leu Ser Ala Gln			
50	55	60	
Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly			
65	70	75	80
Gly Gly Phe Ser Glu Val Ile Phe Arg Leu Glu His His His His His			
85	90	95	

His

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 981 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..981
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

ATG AAG TGT ATA TTA TTT AAA TGG GTA CTG TGC CTG TTA CTG GGT TTT	48		
Met Lys Cys Ile Leu Phe Lys Trp Val Leu Cys Leu Leu Leu Gly Phe			
1	5	10	15
TCT TCG GTA TCC TAT TCC CCG GAG TTT ACG ATA GAC TTT TCG ACC CAA	96		
Ser Ser Val Ser Tyr Ser Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln			
20	25	30	
CAA AGT TAT GTC TCT TCG TTA AAT AGT ATA CGG ACA GAG ATA TCG ACC	144		
Gln Ser Tyr Val Ser Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr			
35	40	45	
CCT CTT GAA CAT ATA TCT CAG GGG ACC ACA TCG GTG TCT GTT ATT AAC	192		
Pro Leu Glu His Ile Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn			
50	55	60	
CAC ACC CAC GGC AGT TAT TTT GCT GTG GAT ATA CGA GGG CTT GAT GTC	240		
His Thr His Gly Ser Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val			
65	70	75	80
TAT CAG GCG CGT TTT GAC CAT CTT CGT CTG ATT ATT GAG CAA AAT AAT	288		
Tyr Gln Ala Arg Phe Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn			
85	90	95	
TTA TAT GTG GCA GGG TTC GTT AAT ACG GCA ACA AAT ACT TTC TAC CGT	336		
Leu Tyr Val Ala Gly Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg			
100	105	110	
TTT TCA GAT TTT ACA CAT ATA TCA GTG CCC GGT GTG ACA ACG GTT TCC	384		
Phe Ser Asp Phe Thr His Ile Ser Val Pro Gly Val Thr Thr Val Ser			
115	120	125	
ATG ACA ACG GAC AGC AGT TAT ACC ACT CTG CAA CGT GTC GCA GCG CTG	432		
Met Thr Thr Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu			
130	135	140	

GAA CGT TCC GGA ATG CAA ATC AGT CGT CAC TCA CTG GTT TCA TCA TAT Glu Arg Ser Gly Met Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr 145 150 155 160	480
CTG GCG TTA ATG GAG TTC AGT GGT AAT ACA ATG ACC AGA GAT GCA TCC Leu Ala Leu Met Glu Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser 165 170 175	528
AGA GCA GTT CTG CGT TTT GTC ACT GTC ACA GCA GAA GCC TTA CGC TTC Arg Ala Val Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe 180 185 190	576
AGG CAG ATA CAG AGA GAA TTT CGT CAG GCA CTG TCT GAA ACT GCT CCT Arg Gln Ile Gln Arg Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro 195 200 205	624
GTG TAT ACG ATG ACG CCG GGA GAC GTG GAC CTC ACT CTG AAC TGG GGG Val Tyr Thr Met Thr Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly 210 215 220	672
CGA ATC AGC AAT GTG CTT CCG GAG TAT CGG GGA GAG GAT GGT GTC AGA Arg Ile Ser Asn Val Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg 225 230 235 240	720
GTG GGG AGA ATA TCC TTT AAT AAT ATA TCA GCG ATA CTG GGG ACT GTG Val Gly Arg Ile Ser Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val 245 250 255	768
GCC GTT ATA CTG AAT TGC CAT CAT CAG GGG GCG CGT TCT GTT CGC GCC Ala Val Ile Leu Asn Cys His His Gln Gly Ala Arg Ser Val Arg Ala 260 265 270	816
GTG AAT GAA GAG AGT CAA CCA GAA TGT CAG ATA ACT GGC GAC AGG CCT Val Asn Glu Glu Ser Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro 275 280 285	864
GTT ATA AAA ATA AAC AAT ACA TTA TGG GAA AGT AAT ACA GCT GCA GCG Val Ile Lys Ile Asn Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala 290 295 300	912
TTT CTG AAC AGA AAG TCA CAG TTT TTA TAT ACA ACG GGT AAA CTC GAG Phe Leu Asn Arg Lys Ser Gln Phe Leu Tyr Thr Thr Gly Lys Leu Glu 305 310 315 320	960
CAC CAC CAC CAC CAC TG His His His His His 325	981

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 326 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Met Lys Cys Ile Leu Phe Lys Trp Val Leu Cys Leu Leu Leu Gly Phe 1 5 10 15
Ser Ser Val Ser Tyr Ser Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln 20 25 30
Gln Ser Tyr Val Ser Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr 35 40 45
Pro Leu Glu His Ile Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn 50 55 60

His Thr His Gly Ser Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val
 65 70 75 80
 Tyr Gln Ala Arg Phe Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn
 85 90 95
 Leu Tyr Val Ala Gly Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg
 100 105 110
 Phe Ser Asp Phe Thr His Ile Ser Val Pro Gly Val Thr Thr Val Ser
 115 120 125
 Met Thr Thr Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu
 130 135 140
 Glu Arg Ser Gly Met Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr
 145 150 155 160
 Leu Ala Leu Met Glu Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser
 165 170 175
 Arg Ala Val Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe
 180 185 190
 Arg Gln Ile Gln Arg Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro
 195 200 205
 Val Tyr Thr Met Thr Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly
 210 215 220
 Arg Ile Ser Asn Val Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg
 225 230 235 240
 Val Gly Arg Ile Ser Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val
 245 250 255
 Ala Val Ile Leu Asn Cys His His Gln Gly Ala Arg Ser Val Arg Ala
 260 265 270
 Val Asn Glu Glu Ser Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro
 275 280 285
 Val Ile Lys Ile Asn Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala
 290 295 300
 Phe Leu Asn Arg Lys Ser Gln Phe Leu Tyr Thr Thr Gly Lys Leu Glu
 305 310 315 320
 His His His His His
 325

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 294 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..294

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ATG AAG AAG ATG TTT ATG GCG GTT TTA TTT GCA TTA GCT TCT GTT AAT
 Met Lys Lys Met Phe Met Ala Val Leu Phe Ala Leu Ala Ser Val Asn
 1 5 10 15

48

GCA ATG GCG GCG GAT TGT GCT AAA GGT AAA ATT GAG TTT TCC AAG TAT Ala Met Ala Ala Asp Cys Ala Lys Gly Lys Ile Glu Phe Ser Lys Tyr 20 25 30	96
AAT GAG GAT GAC ACA TTT ACA GTG AAG GTT GAC GGG AAA GAA TAC TGG Asn Glu Asp Asp Thr Phe Thr Val Lys Val Asp Gly Lys Glu Tyr Trp 35 40 45	144
ACC AGT CGC TGG AAT CTG CAA CCG TTA CTG CAA AGT GCT CAG TTG ACA Thr Ser Arg Trp Asn Leu Gln Pro Leu Leu Gln Ser Ala Gln Leu Thr 50 55 60	192
GGA ATG ACT GTC ACA ATC AAA TCC AGT ACC TGT GAA TCA GGC TCC GGA Gly Met Thr Val Thr Ile Lys Ser Ser Thr Cys Glu Ser Gly Ser Gly 65 70 75 80	240
TTT GCT GAA GTG CAG TTT AAT AAT GAC CTC GAG CAC CAC CAC CAC Phe Ala Glu Val Gln Phe Asn Asn Asp Leu Glu His His His His His 85 90 95	288
CAC TG His	294

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 97 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Met Lys Lys Met Phe Met Ala Val Leu Phe Ala Leu Ala Ser Val Asn 1 5 10 15
Ala Met Ala Ala Asp Cys Ala Lys Gly Lys Ile Glu Phe Ser Lys Tyr 20 25 30
Asn Glu Asp Asp Thr Phe Thr Val Lys Val Asp Gly Lys Glu Tyr Trp 35 40 45
Thr Ser Arg Trp Asn Leu Gln Pro Leu Leu Gln Ser Ala Gln Leu Thr 50 55 60
Gly Met Thr Val Thr Ile Lys Ser Ser Thr Cys Glu Ser Gly Ser Gly 65 70 75 80
Phe Ala Glu Val Gln Phe Asn Asn Asp Leu Glu His His His His His 85 90 95

His

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CGGAATTCAA GGAATTACCG TTAGACTTCT CG

32

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GGCTCGAGTC AACTGCTAAT AGTTCTGC

28

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CGGAATTCCG GGAGTTACG ATAGACTTTT CG

32

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GGCTCGAGTT ATTTACCCGT TGTATATAA

29

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2127 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..2127
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

ATG AAA ATA AAA ACA GGT GCA CGC ATC CTC GCA TTA TCC GCA TTA ACG Met Lys Ile Lys Thr Gly Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr	48		
1	5	10	15

ACG ATG ATG TTT TCC GCC TCG GCT CTC GCC AAA ATC GAA GAA GGT AAA Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys	96	
20	25	30

CTG GTA ATC TGG ATT AAC GGC GAT AAA GGC TAT AAC GGT CTC GCT GAA Leu Val Ile Trp Ile Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu	144	
35	40	45

GTC GGT AAG AAA TTC GAG AAA GAT ACC GGA ATT AAA GTC ACC GTT GAG Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu	192
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50	55	60	
CAT CCG GAT AAA CTG GAA GAG AAA TTC CCA CAG GTT GCG GCA ACT GGC His Pro Asp Lys Leu Glu Glu Lys Phe Pro Gln Val Ala Ala Thr Gly 65 70 75 80			240
GAT GGC CCT GAC ATT ATC TTC TGG GCA CAC GAC CGC TTT GGT GGC TAC Asp Gly Pro Asp Ile Ile Phe Trp Ala His Asp Arg Phe Gly Gly Tyr 85 90 95			288
GCT CAA TCT GGC CTG TTG GCT GAA ATC ACC CCG GAC AAA GCG TTC CAG Ala Gln Ser Gly Leu Leu Ala Glu Ile Thr Pro Asp Lys Ala Phe Gln 100 105 110			336
GAC AAG CTG TAT CCG TTT ACC TGG GAT GCC GTA CGT TAC AAC GGC AAG Asp Lys Leu Tyr Pro Phe Thr Trp Asp Ala Val Arg Tyr Asn Gly Lys 115 120 125			384
CTG ATT GCT TAC CCG ATC GCT GTT GAA GCG TTA TCG CTG ATT TAT AAC Leu Ile Ala Tyr Pro Ile Ala Val Glu Ala Leu Ser Leu Ile Tyr Asn 130 135 140			432
AAA GAT CTG CTG CCG AAC CCG CCA AAA ACC TGG GAA GAG ATC CCG GCG Lys Asp Leu Leu Pro Asn Pro Lys Thr Trp Glu Glu Ile Pro Ala 145 150 155 160			480
CTG GAT AAA GAA CTG AAA GCG AAA GGT AAG AGC GCG CTG ATG TTC AAC Leu Asp Lys Glu Leu Lys Ala Lys Gly Lys Ser Ala Leu Met Phe Asn 165 170 175			528
CTG CAA GAA CCG TAC TTC ACC TGG CCG CTG ATT GCT GCT GAC GGG GGT Leu Gln Glu Pro Tyr Phe Thr Trp Pro Leu Ile Ala Ala Asp Gly Gly 180 185 190			576
TAT GCG TTC AAG TAT GAA AAC GGC AAG TAC GAC ATT AAA GAC GTG GGC Tyr Ala Phe Lys Tyr Glu Asn Gly Lys Tyr Asp Ile Lys Asp Val Gly 195 200 205			624
GTG GAT AAC GCT GGC GCG AAA GCG GGT CTG ACC TTC CTG GTT GAC CTG Val Asp Asn Ala Gly Ala Lys Ala Gly Leu Thr Phe Leu Val Asp Leu 210 215 220			672
ATT AAA AAC AAA CAC ATG AAT GCA GAC ACC GAT TAC TCC ATC GCA GAA Ile Lys Asn Lys His Met Asn Ala Asp Thr Asp Tyr Ser Ile Ala Glu 225 230 235 240			720
GCT GCC TTT AAT AAA GGC GAA ACA GCG ATG ACC ATC AAC GGC CCG TGG Ala Ala Phe Asn Lys Gly Glu Thr Ala Met Thr Ile Asn Gly Pro Trp 245 250 255			768
GCA TGG TCC AAC ATC GAC ACC AGC AAA GTG AAT TAT GGT GTA ACG GTA Ala Trp Ser Asn Ile Asp Thr Ser Lys Val Asn Tyr Gly Val Thr Val 260 265 270			816
CTG CCG ACC TTC AAG GGT CAA CCA TCC AAA CCG TTC GTT GGC GTG CTG Leu Pro Thr Phe Lys Gly Gln Pro Ser Lys Pro Phe Val Gly Val Leu 275 280 285			864
AGC GCA GGT ATT AAC GCC GCC AGT CCG AAC AAA GAG CTG GCG AAA GAG Ser Ala Gly Ile Asn Ala Ala Ser Pro Asn Lys Glu Leu Ala Lys Glu 290 295 300			912

TTC CTC GAA AAC TAT CTG CTG ACT GAT GAA GGT CTG GAA GCG GTT AAT Phe Leu Glu Asn Tyr Leu Leu Thr Asp Glu Gly Leu Ala Val Asn 305 310 315 320	960
AAA GAC AAA CCG CTG GGT GCC GTA GCG CTG AAG TCT TAC GAG GAA GAG Lys Asp Lys Pro Leu Gly Ala Val Ala Leu Lys Ser Tyr Glu Glu 325 330 335	1008
TTG GCG AAA GAT CCA CGT ATT GCC GCC ACC ATG GAA AAC GCC CAG AAA Leu Ala Lys Asp Pro Arg Ile Ala Ala Thr Met Glu Asn Ala Gln Lys 340 345 350	1056
GGT GAA ATC ATG CCG AAC ATC CCG CAG ATG TCC GCT TTC TGG TAT GCC Gly Glu Ile Met Pro Asn Ile Pro Gln Met Ser Ala Phe Trp Tyr Ala 355 360 365	1104
GTG CGT ACT GCG GTG ATC AAC GCC GCC AGC GGT CGT CAG ACT GTC GAT Val Arg Thr Ala Val Ile Asn Ala Ala Ser Gly Arg Gln Thr Val Asp 370 375 380	1152
GAA GCC CTG AAA GAC GCG CAG ACT TCG AGC TCG AAC AAC AAC AAC AAT Glu Ala Leu Lys Asp Ala Gln Thr Ser Ser Ser Asn Asn Asn Asn Asn 385 390 395 400	1200
AAC AAT AAC AAC AAC CTC GGG ATC GAG GGA AGG ATT TCA GAA TTC AAG Asn Asn Asn Asn Leu Gly Ile Glu Gly Arg Ile Ser Glu Phe Lys 405 410 415	1248
GAA TTT ACC TTA GAC TTC TCG ACT GCA AAG ACG TAT GTA GAT TCG CTG Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp Ser Leu 420 425 430	1296
AAT GTC ATT CGC TCT GCA ATA GGT ACT CCA TTA CAG ACT ATT TCA TCA Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile Ser Ser 435 440 445	1344
GGA GGT ACG TCT TTA CTG ATG ATT GAT AGT GGC TCA GGG GAT AAT TTG Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Ser Gly Asp Asn Leu 450 455 460	1392
TTT GCA GTT GAT GTC AGA GGG ATA GAT GCA GAG GAA GGG CGG TTT AAT Phe Ala Val Asp Val Arg Gly Ile Asp Ala Glu Glu Gly Arg Phe Asn 465 470 475 480	1440
AAT CTA CGG CTT ATT GTT GAA CGA AAT AAT TTA TAT GTG ACA GGA TTT Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr Gly Phe 485 490 495	1488
GTT AAC AGG ACA AAT AAT GTT TTT TAT CGC TTT GCT GAT TTT TCA CAT Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe Ser His 500 505 510	1536
GTT ACC TTT CCA GGT ACA ACA GCG GTT ACA TTG TCT GGT GAC AGT AGC Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp Ser Ser 515 520 525	1584
TAT ACC ACG TTA CAG CGT GTT GCA GGG ATC AGT CGT ACG GGG ATG CAG Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly Met Gln 530 535 540	1632
ATA AAT CGC CAT TCG TTG ACT ACT TCT TAT CTG GAT TTA ATG TCG CAT Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met Ser His 545 550 555 560	1680
AGT GGA ACC TCA CTG ACG CAG TCT GTG GCA AGA GCG ATG TTA CGG TTT Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu Arg Phe 565 570 575	1728
GTT ACT GTG ACA GCT GAA GCT TTA CGT TTT CGG CAA ATA CAG AGG GGA Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Gly	1776

580	585	590	
TTT CGT ACA ACA CTG GAT CTC AGT GGG CGT TCT TAT GTC ATG ACT Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val Met Thr 595 600 605			1824
GCT GAA GAT GTT GAT CTT ACA TTG AAC TGG GGA AGG TTG AGT AGC GTC Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser Ser Val 610 615 620			1872
CTG CCT GAC TAT CAT GGA CAA GAC TCT GTT CGT GTC GGA AGA ATT TCT Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg Ile Ser 625 630 635 640			1920
TTT GGA AGC ATT AAT GCA ATT CTG GGA AGC GTG GCA TTA ATA CTG AAT Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile Leu Asn 645 650 655			1968
TGT CAT CAT CAT GCA TCG CGA GTT GCC AGA ATG GCA TCT GAT GAG TTT Cys His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp Glu Phe 660 665 670			2016
CCT TCT ATG TGT CCG GCA GAT GGA AGA GTC CGT GGG ATT ACG CAC AAT Pro Ser Met Cys Pro Ala Asp Gly Arg Val Arg Gly Ile Thr His Asn 675 680 685			2064
AAA ATA TTG TGG GAT TCA TCC ACT CTG GGG GCA ATT CTG ATG CGC AGA Lys Ile Leu Trp Asp Ser Ser Thr Leu Gly Ala Ile Leu Met Arg Arg 690 695 700			2112
ACT ATT AGC AGT TG Thr Ile Ser Ser 705			2127

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 708 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Met Lys Ile Lys Thr Gly Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr 1 5 10 15
Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys 20 25 30
Leu Val Ile Trp Ile Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu 35 40 45
Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu 50 55 60
His Pro Asp Lys Leu Glu Lys Phe Pro Gln Val Ala Ala Thr Gly 65 70 75 80
Asp Gly Pro Asp Ile Ile Phe Trp Ala His Asp Arg Phe Gly Gly Tyr 85 90 95
Ala Gln Ser Gly Leu Leu Ala Glu Ile Thr Pro Asp Lys Ala Phe Gln 100 105 110
Asp Lys Leu Tyr Pro Phe Thr Trp Asp Ala Val Arg Tyr Asn Gly Lys 115 120 125
Leu Ile Ala Tyr Pro Ile Ala Val Glu Ala Leu Ser Leu Ile Tyr Asn

130	135	140
Lys Asp Leu Leu Pro Asn Pro Pro Lys Thr Trp Glu Glu Ile Pro Ala		
145 150 155 160		
Leu Asp Lys Glu Leu Lys Ala Lys Gly Lys Ser Ala Leu Met Phe Asn		
165 170 175		
Leu Gln Glu Pro Tyr Phe Thr Trp Pro Leu Ile Ala Ala Asp Gly Gly		
180 185 190		
Tyr Ala Phe Lys Tyr Glu Asn Gly Lys Tyr Asp Ile Lys Asp Val Gly		
195 200 205		
Val Asp Asn Ala Gly Ala Lys Ala Gly Leu Thr Phe Leu Val Asp Leu		
210 215 220		
Ile Lys Asn Lys His Met Asn Ala Asp Thr Asp Tyr Ser Ile Ala Glu		
225 230 235 240		
Ala Ala Phe Asn Lys Gly Glu Thr Ala Met Thr Ile Asn Gly Pro Trp		
245 250 255		
Ala Trp Ser Asn Ile Asp Thr Ser Lys Val Asn Tyr Gly Val Thr Val		
260 265 270		
Leu Pro Thr Phe Lys Gly Gln Pro Ser Lys Pro Phe Val Gly Val Leu		
275 280 285		
Ser Ala Gly Ile Asn Ala Ala Ser Pro Asn Lys Glu Leu Ala Lys Glu		
290 295 300		
Phe Leu Glu Asn Tyr Leu Leu Thr Asp Glu Gly Leu Glu Ala Val Asn		
305 310 315 320		
Lys Asp Lys Pro Leu Gly Ala Val Ala Leu Lys Ser Tyr Glu Glu Glu		
325 330 335		
Leu Ala Lys Asp Pro Arg Ile Ala Ala Thr Met Glu Asn Ala Gln Lys		
340 345 350		
Gly Glu Ile Met Pro Asn Ile Pro Gln Met Ser Ala Phe Trp Tyr Ala		
355 360 365		
Val Arg Thr Ala Val Ile Asn Ala Ala Ser Gly Arg Gln Thr Val Asp		
370 375 380		
Glu Ala Leu Lys Asp Ala Gln Thr Ser Ser Asn Asn Asn Asn Asn		
385 390 395 400		
Asn Asn Asn Asn Leu Gly Ile Glu Gly Arg Ile Ser Glu Phe Lys		
405 410 415		
Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp Ser Leu		
420 425 430		
Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile Ser Ser		
435 440 445		
Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Ser Gly Asp Asn Leu		
450 455 460		
Phe Ala Val Asp Val Arg Gly Ile Asp Ala Glu Glu Gly Arg Phe Asn		
465 470 475 480		
Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr Gly Phe		
485 490 495		
Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe Ser His		
500 505 510		

Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp Ser Ser
 515 520 525
 Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly Met Gln
 530 535 540
 Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met Ser His
 545 550 555 560
 Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu Arg Phe
 565 570 575
 Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Gly
 580 585 590
 Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val Met Thr
 595 600 605
 Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser Ser Val
 610 615 620
 Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg Ile Ser
 625 630 635 640
 Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile Leu Asn
 645 650 655
 Cys His His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp Glu Phe
 660 665 670
 Pro Ser Met Cys Pro Ala Asp Gly Arg Val Arg Gly Ile Thr His Asn
 675 680 685
 Lys Ile Leu Trp Asp Ser Ser Thr Leu Gly Ala Ile Leu Met Arg Arg
 690 695 700
 Thr Ile Ser Ser
 705

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2136 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..2136

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

ATG AAA ATA AAA ACA GGT GCA CGC ATC CTC GCA TTA TCC GCA TTA ACG Met Lys Ile Lys Thr Gly Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr 1 5 10 15	48
ACG ATG ATG TTT TCC GCC TCG GCT CTC GCC AAA ATC GAA GAA GGT AAA Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys 20 25 30	96
CTG GTA ATC TGG ATT AAC GGC GAT AAA GGC TAT AAC GGT CTC GCT GAA Leu Val Ile Trp Ile Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu 35 40 45	144
GTC GGT AAG AAA TTC GAG AAA GAT ACC GGA ATT AAA GTC ACC GTT GAG Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu 50 55 60	192

CAT CCG GAT AAA CTG GAA GAG AAA TTC CCA CAG GTT GCG GCA ACT GGC His Pro Asp Lys Leu Glu Glu Lys Phe Pro Gln Val Ala Ala Thr Gly 65 70 75 80	240
GAT GGC CCT GAC ATT ATC TTC TGG GCA CAC GAC CGC TTT GGT GGC TAC Asp Gly Pro Asp Ile Ile Phe Trp Ala His Asp Arg Phe Gly Gly Tyr 85 90 95	288
GCT CAA TCT GGC CTG TTG GCT GAA ATC ACC CCG GAC AAA GCG TTC CAG Ala Gln Ser Gly Leu Leu Ala Glu Ile Thr Pro Asp Lys Ala Phe Gln 100 105 110	336
GAC AAG CTG TAT CCG TTT ACC TGG GAT GCC GTA CGT TAC AAC GGC AAG Asp Lys Leu Tyr Pro Phe Thr Trp Asp Ala Val Arg Tyr Asn Gly Lys 115 120 125	384
CTG ATT GCT TAC CCG ATC GCT GTT GAA GCG TTA TCG CTG ATT TAT AAC Leu Ile Ala Tyr Pro Ile Ala Val Glu Ala Leu Ser Leu Ile Tyr Asn 130 135 140	432
AAA GAT CTG CTG CCG AAC CCG CCA AAA ACC TGG GAA GAG ATC CCG GCG Lys Asp Leu Leu Pro Asn Pro Pro Lys Thr Trp Glu Glu Ile Pro Ala 145 150 155 160	480
CTG GAT AAA GAA CTG AAA GCG AAA GGT AAG AGC GCG CTG ATG TTC AAC Leu Asp Lys Glu Leu Lys Ala Lys Gly Lys Ser Ala Leu Met Phe Asn 165 170 175	528
CTG CAA GAA CCG TAC TTC ACC TGG CCG CTG ATT GCT GCT GAC GGG GGT Leu Gln Glu Pro Tyr Phe Thr Trp Pro Leu Ile Ala Ala Asp Gly Gly 180 185 190	576
TAT GCG TTC AAG TAT GAA AAC GGC AAG TAC GAC ATT AAA GAC GTG GGC Tyr Ala Phe Lys Tyr Glu Asn Gly Lys Tyr Asp Ile Lys Asp Val Gly 195 200 205	624
GTG GAT AAC GCT GGC GCG AAA GCG GGT CTG ACC TTC CTG GTT GAC CTG Val Asp Asn Ala Gly Ala Lys Leu Thr Phe Leu Val Asp Leu 210 215 220	672
ATT AAA AAC AAA CAC ATG AAT GCA GAC ACC GAT TAC TCC ATC GCA GAA Ile Lys Asn Lys His Met Asn Ala Asp Thr Asp Tyr Ser Ile Ala Glu 225 230 235 240	720
GCT GCC TTT AAT AAA GGC GAA ACA GCG ATG ACC ATC AAC GGC CCG TGG Ala Ala Phe Asn Lys Gly Glu Thr Ala Met Thr Ile Asn Gly Pro Trp 245 250 255	768
GCA TGG TCC AAC ATC GAC ACC AGC AAA GTG AAT TAT GGT GTA ACG GTA Ala Trp Ser Asn Ile Asp Thr Ser Lys Val Asn Tyr Gly Val Thr Val 260 265 270	816
CTG CCG ACC TTC AAG GGT CAA CCA TCC AAA CCG TTC GTT GGC GTG CTG Leu Pro Thr Phe Lys Gly Gln Pro Ser Lys Pro Phe Val Gly Val Leu 275 280 285	864
AGC GCA GGT ATT AAC GCC GCC AGT CCG AAC AAA GAG CTG GCG AAA GAG Ser Ala Gly Ile Asn Ala Ala Ser Pro Asn Lys Glu Leu Ala Lys Glu 290 295 300	912
TTC CTC GAA AAC TAT CTG CTG ACT GAT GAA GGT CTG GAA GCG GTT AAT Phe Leu Glu Asn Tyr Leu Leu Thr Asp Glu Gly Leu Glu Ala Val Asn 305 310 315 320	960
AAA GAC AAA CCG CTG GGT GCC GTA GCG CTG AAG TCT TAC GAG GAA GAG Lys Asp Lys Pro Leu Gly Ala Val Ala Leu Lys Ser Tyr Glu Glu Glu 325 330 335	1008
TTG GCG AAA GAT CCA CGT ATT GCC ACC ATG GAA AAC GCC CAG AAA Leu Ala Lys Asp Pro Arg Ile Ala Ala Thr Met Glu Asn Ala Gln Lys	1056

340	345	350	
GGT GAA ATC ATG CCG AAC ATC CCG CAG ATG TCC GCT TTC TGG TAT GCC Gly Glu Ile Met Pro Asn Ile Pro Gln Met Ser Ala Phe Trp Tyr Ala 355	360	365	1104
GTG CGT ACT GCG GTG ATC AAC GCC GCC AGC GGT CGT CAG ACT GTC GAT Val Arg Thr Ala Val Ile Asn Ala Ala Ser Gly Arg Gln Thr Val Asp 370	375	380	1152
GAA GCC CTG AAA GAC GCG CAG ACT TCG AGC TCG AAC AAC AAC AAC AAT Glu Ala Leu Lys Asp Ala Gln Thr Ser Ser Asn Asn Asn Asn Asn 385	390	395	1200
AAC AAT AAC AAC AAC CTC GGG ATC GAG GGA AGG ATT TCA GAA TTC CGG Asn Asn Asn Asn Leu Gly Ile Glu Gly Arg Ile Ser Glu Phe Arg 405	410	415	1248
GAG TTT ACG ATA GAC TTT TCG ACC CAA CAA AGT TAT GTC TCT TCG TTA Glu Phe Thr Ile Asp Phe Ser Thr Gln Gln Ser Tyr Val Ser Ser Leu 420	425	430	1296
AAT AGT ATA CGG ACA GAG ATA TCG ACC CCT CTT GAA CAT ATA TCT CAG Asn Ser Ile Arg Thr Glu Ile Ser Thr Pro Leu Glu His Ile Ser Gln 435	440	445	1344
GGG ACC ACA TCG GTG TCT GTT ATT AAC CAC ACC CAC GGC AGT TAT TTT Gly Thr Thr Ser Val Ser Val Ile Asn His Thr His Gly Ser Tyr Phe 450	455	460	1392
GCT GTG GAT ATA CGA GGG CTT GAT GTC TAT CAG GCG CGT TTT GAC CAT Ala Val Asp Ile Arg Gly Leu Asp Val Tyr Gln Ala Arg Phe Asp His 465	470	475	1440
CTT CGT CTG ATT ATT GAG CAA AAT AAT TTA TAT GTG GCA GGG TTC GTT Leu Arg Leu Ile Ile Glu Gln Asn Asn Leu Tyr Val Ala Gly Phe Val 485	490	495	1488
AAT ACG GCA ACA AAT ACT TTC TAC CGT TTT TCA GAT TTT ACA CAT ATA Asn Thr Ala Thr Asn Thr Phe Tyr Arg Phe Ser Asp Phe Thr His Ile 500	505	510	1536
TCA GTG CCC GGT GTG ACA ACG GTT TCC ATG ACA ACG GAC AGC AGT TAT Ser Val Pro Gly Val Thr Val Ser Met Thr Thr Asp Ser Ser Tyr 515	520	525	1584
ACC ACT CTG CAA CGT GTC GCA GCG CTG GAA CGT TCC GGA ATG CAA ATC Thr Thr Leu Gln Arg Val Ala Ala Leu Glu Arg Ser Gly Met Gln Ile 530	535	540	1632
AGT CGT CAC TCA CTG GTT TCA TCA TAT CTG GCG TTA ATG GAG TTC AGT Ser Arg His Ser Leu Val Ser Ser Tyr Leu Ala Leu Met Glu Phe Ser 545	550	555	1680
GGT AAT ACA ATG ACC AGA GAT GCA TCC AGA GCA GTT CTG CGT TTT GTC Gly Asn Thr Met Thr Arg Asp Ala Ser Arg Ala Val Leu Arg Phe Val 565	570	575	1728
ACT GTC ACA GCA GAA GCC TTA CGC TTC AGG CAG ATA CAG AGA GAA TTT Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Glu Phe 580	585	590	1776
CGT CAG GCA CTG TCT GAA ACT GCT CCT GTG TAT ACG ATG ACG CCG GGA Arg Gln Ala Leu Ser Glu Thr Ala Pro Val Tyr Thr Met Thr Pro Gly 595	600	605	1824
GAC GTG GAC CTC ACT CTG AAC TGG GGG CGA ATC AGC AAT GTG CTT CCG Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Ile Ser Asn Val Leu Pro 610	615	620	1872

GAG TAT CGG GGA GAG GAT GGT GTC AGA GTG GGG AGA ATA TCC TTT AAT Glu Tyr Arg Gly Glu Asp Gly Val Arg Val Gly Arg Ile Ser Phe Asn 625 630 635 640	1920
AAT ATA TCA GCG ATA CTG GGG ACT GTG GCC GTT ATA CTG AAT TGC CAT Asn Ile Ser Ala Ile Leu Gly Thr Val Ala Val Ile Leu Asn Cys His 645 650 655	1968
CAT CAG GGG GCG CGT TCT GTT CGC GCC GTG AAT GAA GAG AGT CAA CCA His Gln Gly Ala Arg Ser Val Arg Ala Val Asn Glu Ser Gln Pro 660 665 670	2016
GAA TGT CAG ATA ACT GGC GAC AGG CCT GTT ATA AAA ATA AAC AAT ACA Glu Cys Gln Ile Thr Gly Asp Arg Pro Val Ile Lys Ile Asn Asn Thr 675 680 685	2064
TTA TGG GAA AGT AAT ACA GCT GCA GCG TTT CTG AAC AGA AAG TCA CAG Leu Trp Glu Ser Asn Thr Ala Ala Ala Phe Leu Asn Arg Lys Ser Gln 690 695 700	2112
TTT TTA TAT ACA ACG GGT AAA TA Phe Leu Tyr Thr Thr Gly Lys 705 710	2136

(2) INFORMATION FOR SEQ ID NO:35:

(1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 711 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Met Lys Ile Lys Thr Gly Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr 1 5 10 15
Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys 20 25 30
Leu Val Ile Trp Ile Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu 35 40 45
Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu 50 55 60
His Pro Asp Lys Leu Glu Lys Phe Pro Gln Val Ala Ala Thr Gly 65 70 75 80
Asp Gly Pro Asp Ile Ile Phe Trp Ala His Asp Arg Phe Gly Gly Tyr 85 90 95
Ala Gln Ser Gly Leu Leu Ala Glu Ile Thr Pro Asp Lys Ala Phe Gln 100 105 110
Asp Lys Leu Tyr Pro Phe Thr Trp Asp Ala Val Arg Tyr Asn Gly Lys 115 120 125
Leu Ile Ala Tyr Pro Ile Ala Val Glu Ala Leu Ser Leu Ile Tyr Asn 130 135 140
Lys Asp Leu Leu Pro Asn Pro Pro Lys Thr Trp Glu Glu Ile Pro Ala 145 150 155 160
Leu Asp Lys Glu Leu Lys Ala Lys Gly Lys Ser Ala Leu Met Phe Asn 165 170 175
Leu Gln Glu Pro Tyr Phe Thr Trp Pro Leu Ile Ala Ala Asp Gly Gly 180 185 190

Tyr Ala Phe Lys Tyr Glu Asn Gly Lys Tyr Asp Ile Lys Asp Val Gly
 195 200 205
 Val Asp Asn Ala Gly Ala Lys Ala Gly Leu Thr Phe Leu Val Asp Leu
 210 215 220
 Ile Lys Asn Lys His Met Asn Ala Asp Thr Asp Tyr Ser Ile Ala Glu
 225 230 235 240
 Ala Ala Phe Asn Lys Gly Glu Thr Ala Met Thr Ile Asn Gly Pro Trp
 245 250 255
 Ala Trp Ser Asn Ile Asp Thr Ser Lys Val Asn Tyr Gly Val Thr Val
 260 265 270
 Leu Pro Thr Phe Lys Gly Gln Pro Ser Lys Pro Phe Val Gly Val Leu
 275 280 285
 Ser Ala Gly Ile Asn Ala Ala Ser Pro Asn Lys Glu Leu Ala Lys Glu
 290 295 300
 Phe Leu Glu Asn Tyr Leu Leu Thr Asp Glu Gly Leu Glu Ala Val Asn
 305 310 315 320
 Lys Asp Lys Pro Leu Gly Ala Val Ala Leu Lys Ser Tyr Glu Glu
 325 330 335
 Leu Ala Lys Asp Pro Arg Ile Ala Ala Thr Met Glu Asn Ala Gln Lys
 340 345 350
 Gly Glu Ile Met Pro Asn Ile Pro Gln Met Ser Ala Phe Trp Tyr Ala
 355 360 365
 Val Arg Thr Ala Val Ile Asn Ala Ala Ser Gly Arg Gln Thr Val Asp
 370 375 380
 Glu Ala Leu Lys Asp Ala Gln Thr Ser Ser Ser Asn Asn Asn Asn Asn
 385 390 395 400
 Asn Asn Asn Asn Leu Gly Ile Glu Gly Arg Ile Ser Glu Phe Arg
 405 410 415
 Glu Phe Thr Ile Asp Phe Ser Thr Gln Gln Ser Tyr Val Ser Ser Leu
 420 425 430
 Asn Ser Ile Arg Thr Glu Ile Ser Thr Pro Leu Glu His Ile Ser Gln
 435 440 445
 Gly Thr Thr Ser Val Ser Val Ile Asn His Thr His Gly Ser Tyr Phe
 450 455 460
 Ala Val Asp Ile Arg Gly Leu Asp Val Tyr Gln Ala Arg Phe Asp His
 465 470 475 480
 Leu Arg Leu Ile Ile Glu Gln Asn Asn Leu Tyr Val Ala Gly Phe Val
 485 490 495
 Asn Thr Ala Thr Asn Thr Phe Tyr Arg Phe Ser Asp Phe Thr His Ile
 500 505 510
 Ser Val Pro Gly Val Thr Val Ser Met Thr Thr Asp Ser Ser Tyr
 515 520 525
 Thr Thr Leu Gln Arg Val Ala Ala Leu Glu Arg Ser Gly Met Gln Ile
 530 535 540
 Ser Arg His Ser Leu Val Ser Ser Tyr Leu Ala Leu Met Glu Phe Ser
 545 550 555 560
 Gly Asn Thr Met Thr Arg Asp Ala Ser Arg Ala Val Leu Arg Phe Val

565	570	575	
Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Glu Phe			
580	585	590	
Arg Gln Ala Leu Ser Glu Thr Ala Pro Val Tyr Thr Met Thr Pro Gly			
595	600	605	
Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Ile Ser Asn Val Leu Pro			
610	615	620	
Glu Tyr Arg Gly Glu Asp Gly Val Arg Val Gly Arg Ile Ser Phe Asn			
625	630	635	640
Asn Ile Ser Ala Ile Leu Gly Thr Val Ala Val Ile Leu Asn Cys His			
645	650	655	
His Gln Gly Ala Arg Ser Val Arg Ala Val Asn Glu Glu Ser Gln Pro			
660	665	670	
Glu Cys Gln Ile Thr Gly Asp Arg Pro Val Ile Lys Ile Asn Asn Thr			
675	680	685	
Leu Trp Glu Ser Asn Thr Ala Ala Ala Phe Leu Asn Arg Lys Ser Gln			
690	695	700	
Phe Leu Tyr Thr Thr Gly Lys			
705	710		

(2) INFORMATION FOR SEQ ID NO:36:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 981 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..981

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT GGT TTC GCT	48		
Met Lys Lys Thr Ala Ile Ala Ile Val Ala Leu Ala Gly Phe Ala			
1	5	10	15
ACC GTT GCG CAA GCT GAC TAC AAG GAC GAC GAT GAC AAG AAG CTT GAA	96		
Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Asp Lys Lys Leu Glu			
20	25	30	
TTC AAG GAA TTT ACC TTA GAC TTC TCG ACT GCA AAG ACG TAT GTA GAT	144		
Phe Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp			
35	40	45	
TCG CTG AAT GTC ATT CGC TCT GCA ATA GGT ACT CCA TTA CAG ACT ATT	192		
Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile			
50	55	60	
TCA TCA GGA GGT ACG TCT TTA CTG ATG ATT GAT AGT GGC TCA GGG GAT	240		
Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Ser Gly Asp			
65	70	75	80
AAT TTG TTT GCA GTT GAT GTC AGA GGG ATA GAT GCA GAG GAA GGG CGG	288		
Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp Ala Glu Glu Gly Arg			
85	90	95	
TTT AAT AAT CTA CGG CTT ATT GTT GAA CGA AAT AAT TTA TAT GTG ACA	336		

Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr			
100	105	110	
GGA TTT GTT AAC AGG ACA AAT AAT GTT TTT TAT CGC TTT GCT GAT TTT			384
Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe			
115	120	125	
TCA CAT GTT ACC TTT CCA GGT ACA ACA GCG GTT ACA TTG TCT GGT GAC			432
Ser His Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp			
130	135	140	
AGT AGC TAT ACC ACG TTA CAG CGT GTT GCA GGG ATC AGT CGT ACG GGG			480
Ser Ser Tyr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly			
145	150	155	160
ATG CAG ATA AAT CGC CAT TCG TTG ACT ACT TCT TAT CTG GAT TTA ATG			528
Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met			
165	170	175	
TCG CAT AGT GGA ACC TCA CTG ACG CAG TCT GTG GCA AGA GCG ATG TTA			576
Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu			
180	185	190	
CGG TTT GTT ACT GTG ACA GCT GAA GCT TTA CGT TTT CGG CAA ATA CAG			624
Arg Phe Val Thr Val Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln			
195	200	205	
AGG GGA TTT CGT ACA ACA CTG GAT GAT CTC AGT GGG CGT TCT TAT GTA			672
Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val			
210	215	220	
ATG ACT GCT GAA GAT GTT GAT CTT ACA TTG AAC TGG GGA AGG TTG AGT			720
Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser			
225	230	235	240
AGC GTC CTG CCT GAC TAT CAT GGA CAA GAC TCT GTT CGT GTA GGA AGA			768
Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg			
245	250	255	
ATT TCT TTT GGA AGC ATT AAT GCA ATT CTG GGA AGC GTG GCA TTA ATA			816
Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile			
260	265	270	
CTG AAT TGT CAT CAT GCA TCG CGA GTT GCC AGA ATG GCA TCT GAT			864
Leu Asn Cys His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp			
275	280	285	
GAG TTT CCT TCT ATG TGT CCG GCA GAT GGA AGA GTC CGT GGG ATT ACG			912
Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg Val Arg Gly Ile Thr			
290	295	300	
CAC AAT AAA ATA TTG TGG GAT TCA TCC ACT CTG GGG GCA ATT CTG ATG			960
His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu Gly Ala Ile Leu Met			
305	310	315	320
CGC AGA ACT ATT AGC AGT TG			981
Arg Arg Thr Ile Ser Ser			
325			

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 326 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala
 1 5 10 15

Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Asp Lys Lys Leu Glu
 20 25 30

Phe Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp
 35 40 45

Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile
 50 55 60

Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Ser Gly Asp
 65 70 75 80

Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp Ala Glu Glu Gly Arg
 85 90 95

Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr
 100 105 110

Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe
 115 120 125

Ser His Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp
 130 135 140

Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly
 145 150 155 160

Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met
 165 170 175

Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu
 180 185 190

Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln
 195 200 205

Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val
 210 215 220

Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser
 225 230 235 240

Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg
 245 250 255

Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile
 260 265 270

Leu Asn Cys His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp
 275 280 285

Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg Val Arg Gly Ile Thr
 290 295 300

His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu Gly Ala Ile Leu Met
 305 310 315 320

Arg Arg Thr Ile Ser Ser
 325

(2: INFORMATION FOR SEQ ID NO:38:

- (1) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 990 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..990

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT GGT TTC GCT Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala 1 5 10 15	48
ACC GTT GCG CAA GCT GAC TAC AAG GAC GAC GAT GAC AAG AAG CTT GAA Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Asp Lys Lys Leu Glu 20 25 30	96
TTC CGG GAG TTT ACG ATA GAC TTT TCG ACC CAA CAA AGT TAT GTC TCT Phe Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln Gln Ser Tyr Val Ser 35 40 45	144
TCG TTA AAT AGT ATA CGG ACA GAG ATA TCG ACC CCT CTT GAA CAT ATA Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr Pro Leu Glu His Ile 50 55 60	192
TCT CAG GGG ACC ACA TCG GTG TCT GTT ATT AAC CAC ACC CAC GGC AGT Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn His Thr His Gly Ser 65 70 75 80	240
TAT TTT GCT GTG GAT ATA CGA GGG CTT GAT GTC TAT CAG GCG CGT TTT Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val Tyr Gln Ala Arg Phe 85 90 95	288
GAC CAT CTT CGT CTG ATT ATT GAG CAA AAT AAT TTA TAT GTG GCA GGG Asp His Leu Arg Leu Ile Glu Gln Asn Asn Leu Tyr Val Ala Gly 100 105 110	336
TTC GTT AAT ACG GCA ACA AAT ACT TTC TAC CGT TTT TCA GAT TTT ACA Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg Phe Ser Asp Phe Thr 115 120 125	384
CAT ATA TCA GTG CCC GGT GTG ACA ACG GTT TCC ATG ACA ACG GAC AGC His Ile Ser Val Pro Gly Val Thr Thr Val Ser Met Thr Thr Asp Ser 130 135 140	432
AGT TAT ACC ACT CTG CAA CGT GTC GCA GCG CTG GAA CGT TCC GGA ATG Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu Glu Arg Ser Gly Met 145 150 155 160	480
CAA ATC AGT CGT CAC TCA CTG GTT TCA TCA TAT CTG GCG TTA ATG GAG Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr Leu Ala Leu Met Glu 165 170 175	528
TTC AGT GGT AAT ACA ATG ACC AGA GAT GCA TCC AGA GCA GTT CTG CGT Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser Arg Ala Val Leu Arg 180 185 190	576
TTT GTC ACT GTC ACA GCA GAA GCC TTA CGC TTC AGG CAG ATA CAG AGA Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg 195 200 205	624
GAA TTT CGT CAG GCA CTG TCT GAA ACT GCT CCT GTG TAT ACG ATG ACG Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro Val Tyr Thr Met Thr 210 215 220	672
CCG GGA GAC GTG GAC CTC ACT CTG AAC TGG GGG CGA ATC AGC AAT GTG Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Ile Ser Asn Val 225 230 235 240	720
CTT CCG GAG TAT CGG GGA GAG GAT GGT GTC AGA GTG GGG AGA ATA TCC Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg Val Gly Arg Ile Ser	768

245	250	255	
TTT AAT AAT ATA TCA GCG ATA CTG GGG ACT GTG GCC GTT ATA CTG AAT			
Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val Ala Val Ile Leu Asn			
260	265	270	
TGC CAT CAT CAG GGG GCG CGT TCT GTT CGC GCC GTG AAT GAA GAG AGT			816
Cys His His Gln Gly Ala Arg Ser Val Arg Ala Val Asn Glu Glu Ser			
275	280	285	
CAA CCA GAA TGT CAG ATA ACT GGC GAC AGG CCT GTT ATA AAA ATA AAC			864
Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro Val Ile Lys Ile Asn			
290	295	300	
AAT ACA TTA TGG GAA AGT AAT ACA GCT GCA GCG TTT CTG AAC AGA AAG			912
Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala Phe Leu Asn Arg Lys			
305	310	315	320
TCA CAG TTT TTA TAT ACA ACG GGT AAA TA			990
Ser Gln Phe Leu Tyr Thr Thr Gly Lys			
325	330		

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 329 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Met Lys Lys Thr Ala Ile Ala Ile Val Ala Leu Ala Gly Phe Ala			
1	5	10	15
Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Asp Lys Lys Leu Glu			
20	25	30	
Phe Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln Gln Ser Tyr Val Ser			
35	40	45	
Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr Pro Leu Glu His Ile			
50	55	60	
Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn His Thr His Gly Ser			
65	70	75	80
Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val Tyr Gln Ala Arg Phe			
85	90	95	
Asp His Leu Arg Leu Ile Glu Gln Asn Asn Leu Tyr Val Ala Gly			
100	105	110	
Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg Phe Ser Asp Phe Thr			
115	120	125	
His Ile Ser Val Pro Gly Val Thr Thr Val Ser Met Thr Thr Asp Ser			
130	135	140	
Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu Glu Arg Ser Gly Met			
145	150	155	160
Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr Leu Ala Leu Met Glu			
165	170	175	
Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser Arg Ala Val Leu Arg			
180	185	190	
Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg			

195	200	205
Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro Val Tyr Thr Met Thr		
210	215	220
Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Ile Ser Asn Val		
225	230	235
240		
Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg Val Gly Arg Ile Ser		
245	250	255
Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val Ala Val Ile Leu Asn		
260	265	270
Cys His His Gln Gly Ala Arg Ser Val Arg Ala Val Asn Glu Glu Ser		
275	280	285
Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro Val Ile Lys Ile Asn		
290	295	300
Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala Phe Leu Asn Arg Lys		
305	310	315
320		
Ser Gln Phe Leu Tyr Thr Thr Gly Lys		
325		

CLAIMS

What is claimed is:

5 1. A method of treatment comprising:
 a) providing:
 i) antitoxin directed against at least a portion of an *Escherichia coli* verotoxin in an aqueous solution in therapeutic amount that is administrable.
 and
10 ii) an intoxicated subject; and
 b) administering said antitoxin to said subject.

2. The method of Claim 1 wherein said *Escherichia coli* verotoxin is recombinant.

15 3. The method of Claim 1 wherein said antitoxin is an avian antitoxin.

4. The method of Claim 2 wherein said recombinant *Escherichia coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT1 sequence.

20 5. The method of Claim 2 wherein said recombinant *Escherichia coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT2 sequence.

25 6. The method of Claim 1 wherein said subject is an adult.

7. The method of Claim 1 wherein said subject is a child.

8. The method of Claim 1 wherein said administering is parenteral.

30 9. The method of Claim 1 wherein said administering is oral.

10. A method of prophylactic treatment comprising:

- a) providing:
 - i) an antitoxin directed against at least one *Escherichia coli* verotoxin in an aqueous solution in therapeutic amount that is parenterally administrable, and
 - ii) at least one subject is at risk of diarrheal disease; and
- b) parenterally administering said antitoxin to said subject.

5

11. The method of Claim 10, wherein said subject is at risk of developing extra-intestinal complications of *Escherichia coli* infection.

10

12. The method of Claim 11, wherein said extra-intestinal complication is hemolytic uremic syndrome.

15

13. A composition comprising neutralizing antitoxin directed against at least one *Escherichia coli* verotoxin in an aqueous solution in therapeutic amounts.

14. The composition of Claim 13 wherein said *Escherichia coli* verotoxin is a recombinant toxin.

20

15. The composition of Claim 14 wherein said recombinant *Escherichia coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT1 sequence.

25

16. The composition of Claim 14 wherein said recombinant *Escherichia coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT2 sequence.

30

17. The composition of Claim 14 wherein said antitoxin is directed against a portion of at least one *Escherichia coli* verotoxin.

18. The composition of Claim 14 wherein said portion of *Escherichia coli* is selected from the group consisting of subunit A and subunit B of VT1.

19. The composition of Claim 14 wherein said portion of *Escherichia coli* is selected from the group consisting of subunit A and subunit B of VT2.

20. The composition of Claim 14 wherein said antitoxin is directed against a portion of at least one *Escherichia coli* verotoxin.

21. The composition of Claim 14 wherein said antitoxin is an avian antitoxin.

22. A method of treatment of enteric bacterial infections comprising:

10 a) providing:

i) an avian antitoxin directed against at least one verotoxin produced by *Escherichia coli* in an aqueous solution in therapeutic amount that is parenterally administrable, and

ii) at least one infected subject; and

15 b) parenterally administering said avian antitoxin to said subject.

23. The method of Claim 18 wherein said *Escherichia coli* is selected from the group consisting of *Escherichia coli* serotypes O157:H7; O1:NM; O2:H5; O2:H7; O4:NM; O4:H10; O5:NM; O5:H16; O6:H1; O18:NM; O18:H7; O25:NM; O26:NM; O26:H11; O26:H32; O38:H21; O39:H4; O45:H2; O50:H7; O55:H7; O55:H10; O82:H8; O84:H2; O91:NM; O91:H21; O103:H2; O111:NM; O111:H8; O111:H30; O111:H34; O113:H7; O113:H21; O114:H48; O115:H10; O117:H4; O118:H12; O118:H30; O121:NM; O121:H19; O125:NM; O125:H8; O126:NM; O126:H8; O128:NM; O128:H2; O128:H8; O128:H12; O128:H25; O145:NM; O125:H25; O146:H21; O153:H25; O157:NM; O163:H19; O165:NM; O165:19; and O165:H25

24. The method of Claim 22 wherein said antitoxin comprises antitoxin directed against at least one *Escherichia coli* verotoxin.

30 25. The method of Claim 22 wherein said antitoxin is cross-reactive with at least one *Escherichia coli* verotoxin.

26. The method of Claim 22 wherein said antitoxin is reactive against toxins produced by members of the genus *Shigella*.

27. The method of Claim 26, wherein said antitoxin is reactive against toxins produced by *Shigella dysenteriae*.

28. A method for detecting *Escherichia coli* verotoxin in a sample comprising:

- a) providing:
 - i) a sample;
 - ii) an antitoxin raised against *Escherichia coli* verotoxin; and
 - iii) a reporter reagent capable of binding said antitoxin; and
- b) adding said antitoxin to said sample so that said antitoxin binds to the *Escherichia coli* verotoxin in said sample.

29. The method of Claim 28, wherein said antitoxin is an avian antitoxin.

30. The method of Claim 28, further comprising the steps of:

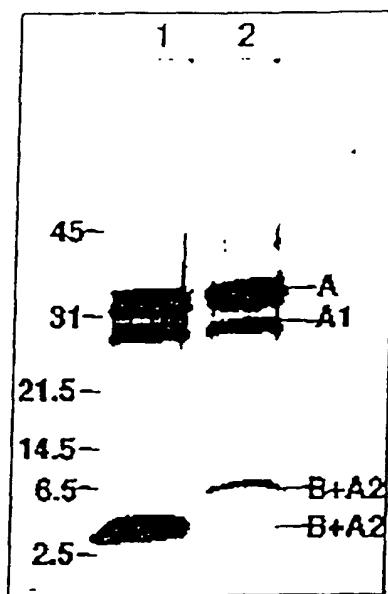
- c) washing said unbound antitoxin from said sample;
- d) adding said reporter reagent to said sample so that said reporter reagent binds to said bound antitoxin;
- e) washing said unbound reporter reagent from said sample; and
- f) detecting said reporter reagent bound to said antitoxin bound to the *Escherichia coli* verotoxin so that the verotoxin is detected.

31. The method of Claim 30 wherein said detecting is selected from the group consisting of enzyme immunoassay, radioimmunoassay, fluorescence immunoassay, flocculation, particle agglutination, and *in situ* chromogenic assay.

32. The method of Claim 30 wherein said sample is a biological sample.

33. The method of Claim 30 wherein said sample is an environmental sample.

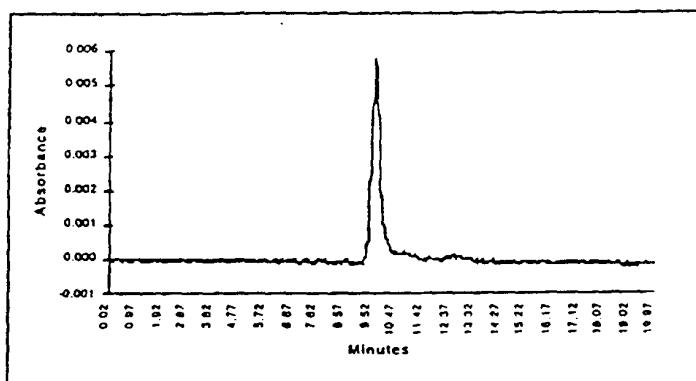
Figure 1.
SDS-PAGE of rVT1 and rVT2



rVT1 (Lane 1) and rVT2 (Lane 2). Positions of molecular weight markers (Kda) are shown at the left. VT component polypeptides are identified at the right.

Figure 2.

HPLC of rVT1



HPLC of rVT2

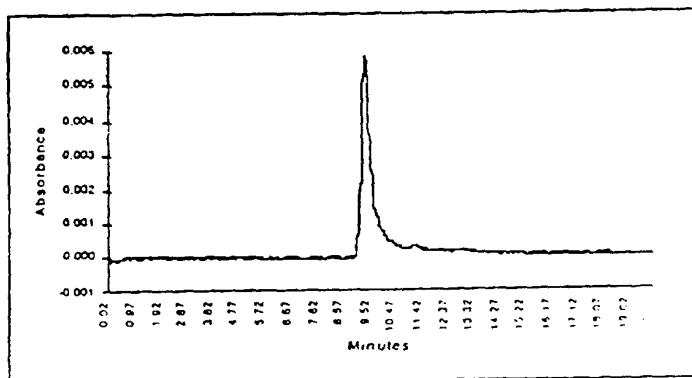


Figure 3.
rVT1 and rVT2 Toxicity in Vero Cell Culture

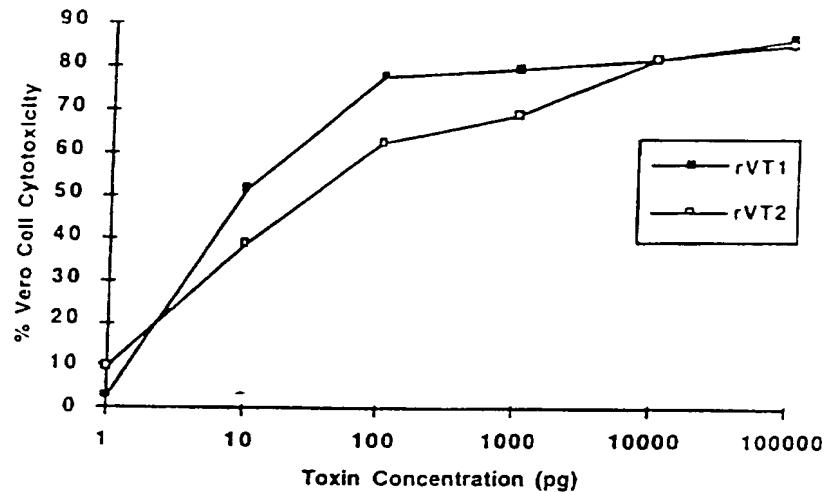


Figure 4.
ELA Reactivity of rVT1 and rVT2 Antibodies to rVT1

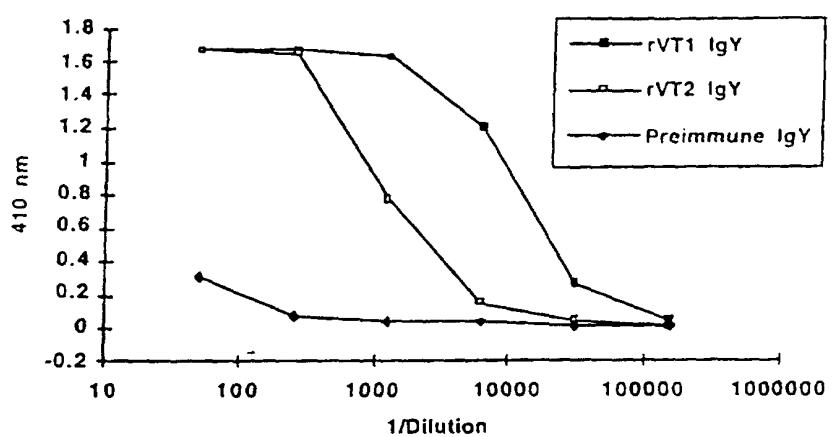


Figure 5.
EIA Reactivity of rVT1 and rVT2 Antibodies to rVT2

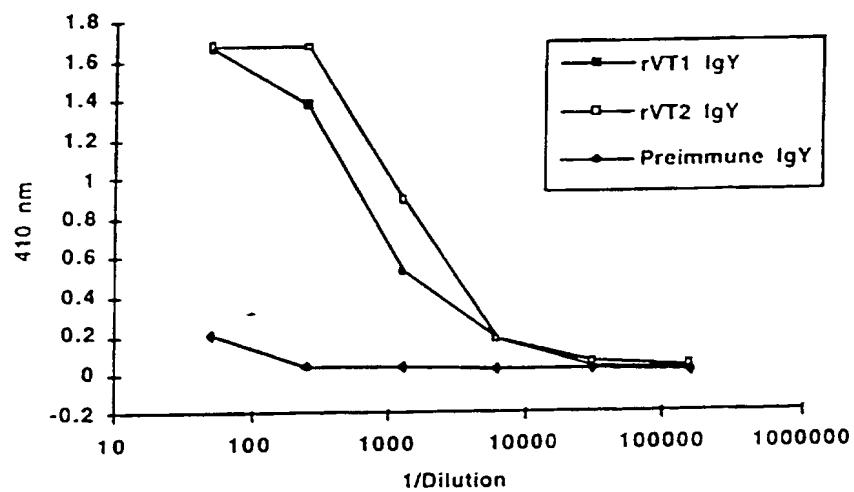
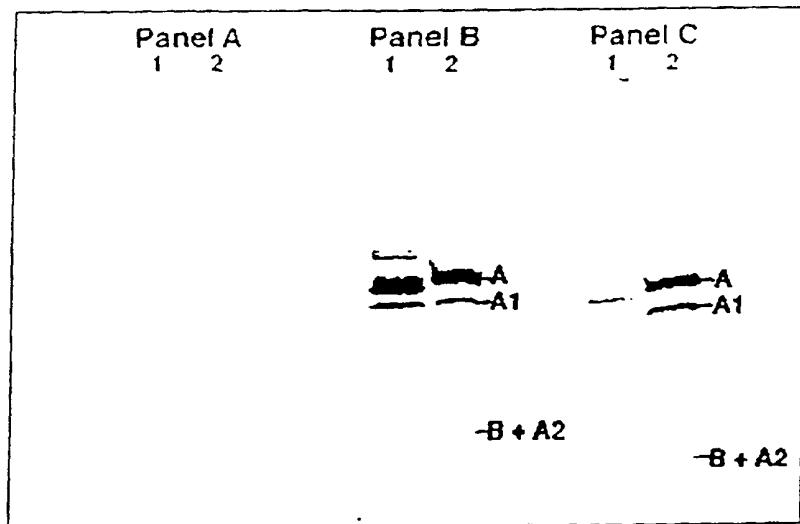


Figure 6.
Western Blot Reactivity of rVT1 and rVT2 Antibodies to rVT's



In this Figure, Panel A contains preimmune IgY, Panel B contains rVT1 IgY, and Panel C contains rVT2 IgY. Lane 1 in each panel contains rVT1 (2 μ g) and Lane 2 contains rVT2 (2 μ g).

Figure 7.
Neutralization of rVT1 Cytotoxicity in Vero Cells

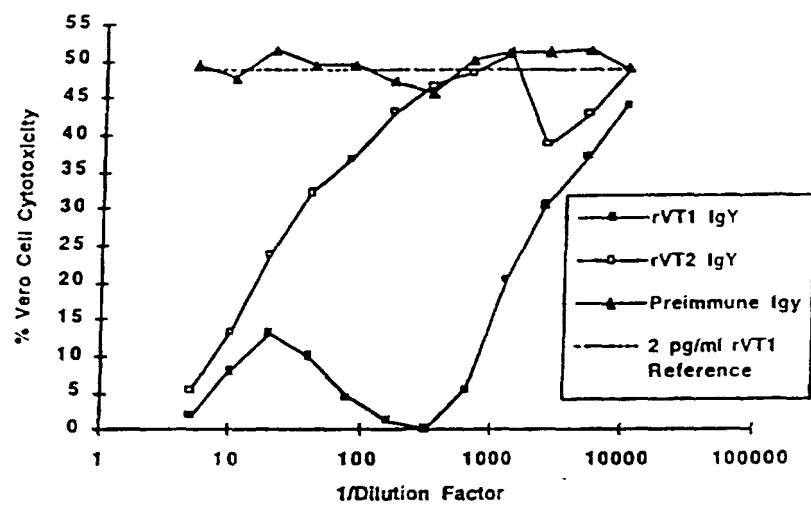


Figure 8.
Neutralization of rVT2 Cytotoxicity in Vero Cells

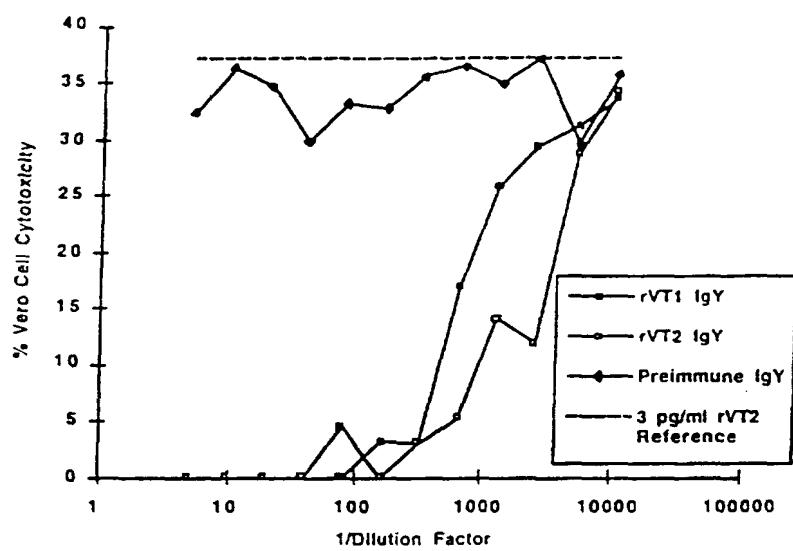
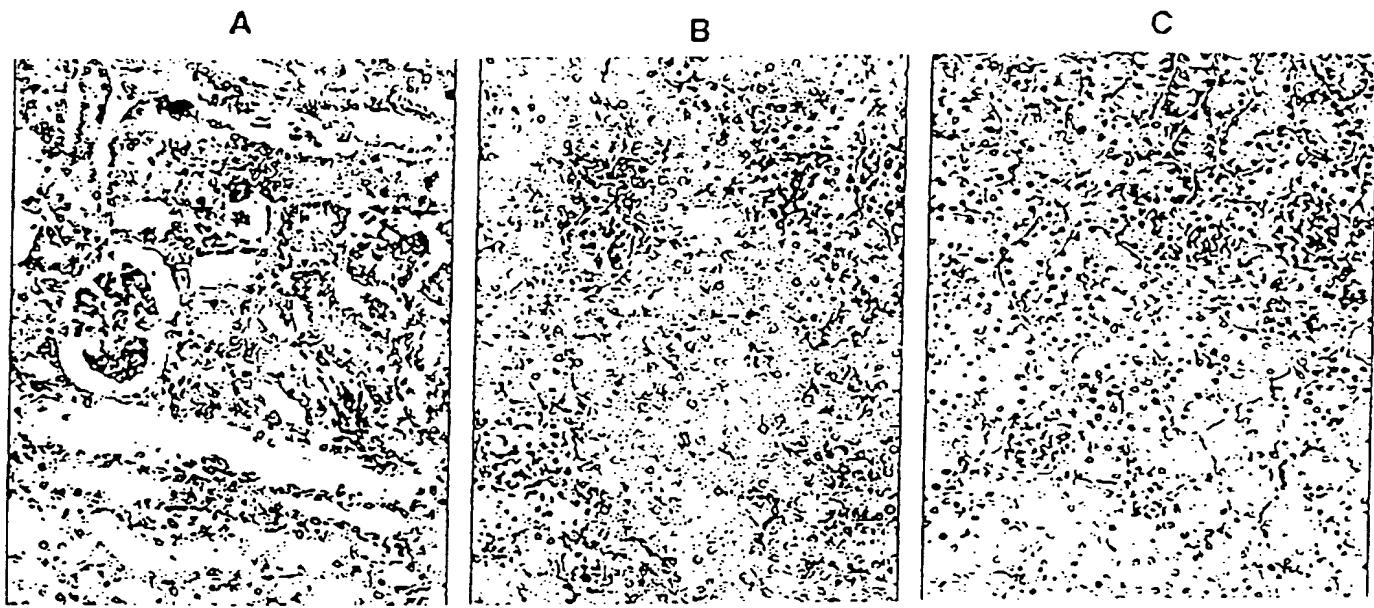
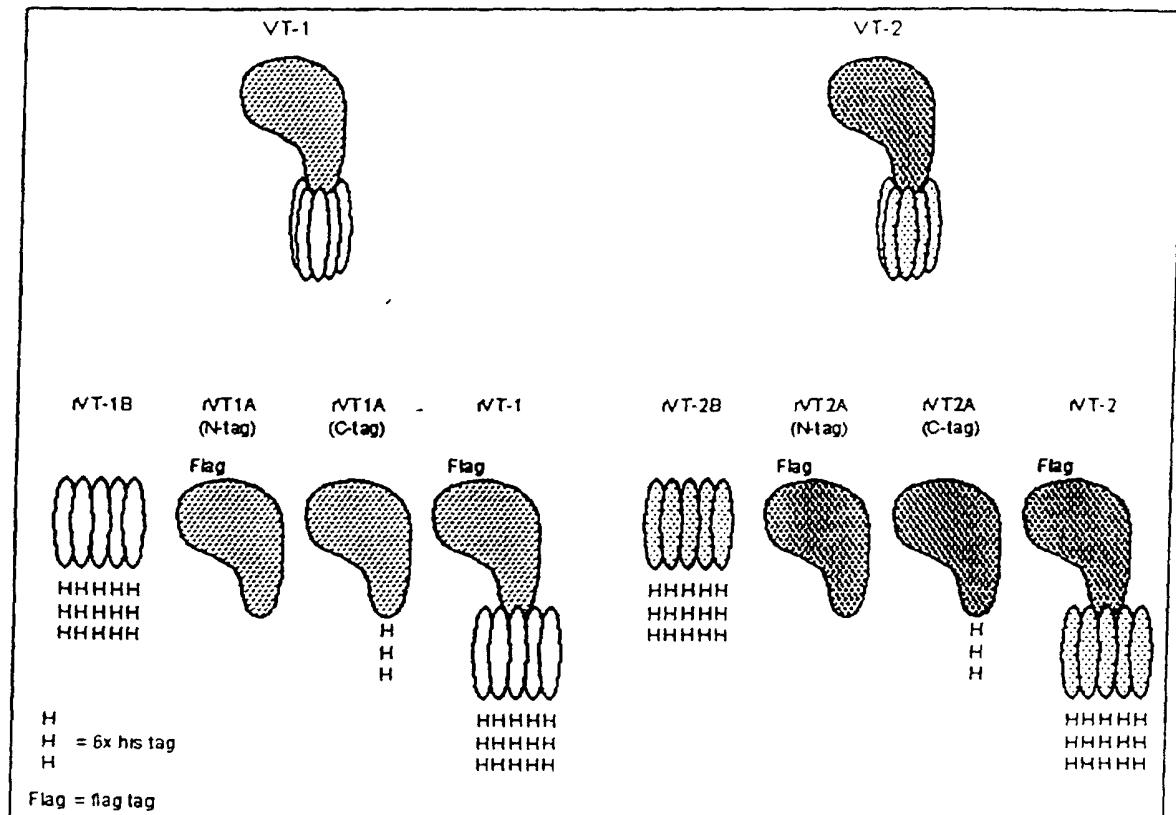


Figure 9.

Renal Sections from *E. coli* O157:H7-Infected Mice Treated with IgY

Representative kidney sections from mice treated with preimmune (Panel A), rVT1 (Panel B) or rVT2 (Panel C) IgY 4 hrs. after infection.

Figure 10.
Fusion Constructs of VT Components and Affinity Tags



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/04093

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) A61K 39/00, 39/02; G01N 35/537

US CL .Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/134.1, 141.1, 150.1, 157.1, 164.1, 169.1, 192.1, 200.1, 236.1, 241.1, 801, 804, 809, 826; 435/7.37; 436/538, 542, 543-547

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BOYD et al. Serological Responses to the B Subunit of Shiga-Like Toxin 1 and Its Peptide Fragments Indicate that the B Subunit Is a Vaccine Candidate To Counter the Action of the Toxin. Infection and Immunity. March 1991, Vol. 59, No. 3, pages 750-757.	1-33
Y	US 5,326,559 A (MILLER) 05 July 1994, columns 4-7.	1-33
X ---	US 5,164,298 A (LINGWOOD et al) 17 November 1992, columns 10-13.	28, 30, 31, 32, 33
Y		-----
Y	US 4,748,018 A (STOLLE et al) 31 May 1988, column 4, lines 25-55.	1-27 and 29 3, 21, 22, 29

Further documents are listed in the continuation of Box C. See patent family annex

* Special categories of cited documents	*P*	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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U document which may throw doubts on priority claims; or which is cited to establish the publication date of another citation or other special reasons as specified	*Y*	document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed	*&*	document member of the same patent family

Date of the actual completion of the international search

10 JULY 1996

Date of mailing of the international search report

27 AUG 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Faxsimile No. (703) 305-3230

Authorized officer

RACHEL FREED

Telephone No. (703) 305-0196

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/US96/04093

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,550,019 A (POLSON) 29 October 1985, column 4, lines 46-68.	3, 21, 22, 29
Y	US 5,204,097 A (ARNON et al) 20 April 1993, column 2, lines 1-16, column 3, lines 33-56 and column 5, lines 53-67.	2 and 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/04093

A. CLASSIFICATION OF SUBJECT MATTER:
US CL

424/134.1, 141.1, 150.1, 157.1, 164.1, 169.1, 192.1, 200.1, 236.1, 241.1, 801, 804, 809, 826; 435/7.37; 436/538, 542, 543-547

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

BIOSIS, MEDLINE, APS

search terms: verotoxin, verocytotoxin, shiga, rvt1, rvt2, rslt1 or rslt2, vaccin? or treat?, recombinant





INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 39/00, 39/02, G01N 35/537		A1	(11) International Publication Number: WO 96/30043 (43) International Publication Date: 3 October 1996 (03.10.96)
(21) International Application Number:	PCT/US96/04093	(81) Designated States:	AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).
(22) International Filing Date:	25 March 1996 (25.03.96)		
(30) Priority Data:	08/410,058 24 March 1995 (24.03.95) US		
(71) Applicant:	OPHIDIAN PHARMACEUTICALS [US/US]; 5445 East Cheryl Parkway, Madison, WI 53711 (US).		
(72) Inventors:	CARROLL, Sean, B.; 3066 Streb Way, Cottage Grove, WI 53527 (US). STAFFORD, Douglas, C.; 21 Laramie Court, Madison, WI 53719 (US). PADHYE, Nisha, V.; 5743 Timber View Court, Fitchburg, WI 53711 (US).		
(74) Agents:	CARROLL, Peter, G. et al.; Medlen & Carroll, Suite 2200, 220 Montgomery Street, San Francisco, CA 94104 (US).		

(54) Title: TREATMENT FOR VEROTOXIN-PRODUCING ESCHERICHIA COLI

(57) Abstract

The present invention includes methods for generating neutralizing antitoxin directed against verotoxins. In particular, the antitoxin directed against these toxins is produced in avian species using soluble recombinant verotoxin proteins. This avian antitoxin is designed so as to be administrable in therapeutic amounts and may be in any form (i.e., as a solid or in aqueous solution). These antitoxins are useful in the treatment of humans and other animals intoxicated with at least one bacterial toxin, as well as for diagnostic assays to detect the presence of toxin in a sample.

FOR THE PURPOSES OF INFORMATION ONLY

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GA	Gabon			VN	Viet Nam

TREATMENT FOR VEROTOXIN-PRODUCING *ESCHERICHIA COLI***FIELD OF THE INVENTION**

The present invention relates to antitoxin therapy for humans and other animals, and
5 diagnostic assays to detect toxins. Antitoxins which neutralize the pathologic effects of
Escherichia coli toxins, such as verotoxin are provided.

BACKGROUND OF THE INVENTION**A. *Escherichia coli* as a Pathogenic Organism**

10 *Escherichia coli* is the organism most commonly isolated in clinical microbiology laboratories, as it is usually present as normal flora in the intestines of humans and other animals. However, it is an important cause of intestinal, as well as extraintestinal infections. For example, in a 1984 survey of nosocomial infections in the United States, *E. coli* was associated with 30.7% of the urinary tract infections, 11.5% of the surgical wound infections, 15 6.4% of the lower respiratory tract infections, 10.5% of the primary bacteremia cases, 7.0% of the cutaneous infections, and 7.4% of the other infections (J.J. Farmer and M.T. Kelly, "Enterobacteriaceae." in Manual of Clinical Microbiology, Balows *et al.*(eds). American Society for Microbiology, [1991], p. 365). Surveillance reports from England, Wales and Ireland for 1986 indicate that *E. coli* was responsible for 5,473 cases of bacteremia (including blood, bone marrow, spleen and heart specimens): of these, 568 were fatal. For spinal fluid specimens, there were 58 cases, with 10 fatalities (J.J. Farmer and M.T. Kelly, "Enterobacteriaceae." in Manual of Clinical Microbiology, Balows *et al.*(eds). American Society for Microbiology, [1991], p. 366). There are no similar data for United States, as these are not reportable diseases in this country.

25 Studies in various countries have identified certain serotypes (based on both the O and H antigens) that are associated with the four major groups of *E. coli* recognized as enteric pathogens. Table 1 lists common serotypes included within these groups. The first group includes the classical enteropathogenic serotypes ("EPEC"); the next group includes those that produce heat-labile or heat-stable enterotoxins ("ETEC"); the third group includes the enteroinvasive strains ("EIEC") that mimic *Shigella* strains in their ability to invade and multiply within intestinal epithelial cells; and the fourth group includes strains and serotypes that cause hemorrhagic colitis or produce Shiga-like toxins (or verotoxins) ("VTEC" or "EHEC" [enterohemorrhagic *E. coli*]).

Table 1.
Pathogenic *E. coli* Serotypes

Group	Associated Serotypes
5	O6:H16; O8:NM; O8:H9; O11:H27; O15:H11; O20:NM; O25:NM; O25:H42; O27:H7; O27:H20; O63:H12; O78:H11; O78:H12; O85:H7; O114:H21; O115:H21; O126:H9; O128ac:H7; O128ac:H12; O128ac:H21; O148:H28; O149:H4; O159:H4; O159:H20; O166:H27; and O167:H5
	O26:NM; O26:H11; O55:NM; O55:H6; O86:NM; O86:H2; O86:H34; O111ab:NM; O111ab:H2; O111ab:H12; O111ab:H21; O114:H2; O119:H6; O125ac:H21; O127:NM; O127:H6; O127:H9; O127:H21; O128ab:H2; O142:H6; and O158:H23
	O28ac:NM; O29:NM; O112ac:NM; O115:NM; O124:NM; O124:H7; O124:H30; O135:NM; O136:NM; O143:NM; O144:NM; O152:NM; O164:NM; and O167:NM
10	O1:NM; O2:H5; O2:H7; O4:NM; O4:H10; O5:NM; O5:H16; O6:H1; O18:NM; O18:H7; O25:NM; O26:NM; O26:H11; O26:H32; O38:H21; O39:H4; O45:H2; O50:H7; O55:H7; O55:H10; O82:H8; O84:H2; O91:NM; O91:H21; O103:H2; O111:NM; O111:H8; O111:H30; O111:H34; O113:H7; O113:H21; O114:H48; O115:H10; O117:H4; O118:H12; O118:H30; O121:NM; O121:H19; O125:NM; O125:H8; O126:NM; O126:H8; O128:NM; O128:H2; O128:H8; O128:H12; O128:H25; O145:NM; O125:H25; O146:H21; O153:H25; O157:NM; O157:H7; O163:H19; O165:NM; O165:19; and O165:H25

B. Verotoxin Producing Strains of *E. coli*

15 Although all of these disease-associated serotypes cause potentially life-threatening disease, *E. coli* O157:H7 and other verotoxin-producing strains have recently gained widespread public attention in the United States due to their recently recognized association with two serious extraintestinal diseases, hemolytic uremic syndrome ("HUS") and thrombotic thrombocytopenic purpura ("TTP"). Worldwide, *E. coli* O157:H7 and other verotoxin-producing *E. coli* (VTEC) are an increasingly important human health problem. First identified as a cause of human illness in early 1982 following two outbreaks of food-related hemorrhagic colitis in Oregon and Michigan (M.A. Karmali, "Infection by Verocytotoxin-Producing *Escherichia coli*," Clin. Microbiol. Rev., 2:15-38 [1989]; and L. W. Riley, *et al.* "Hemorrhagic colitis associated with a rare *Escherichia coli* serotype," New Eng. J. Med.,

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308: 681-685 [1983]), the reported incidence of VTEC-associated disease has risen steadily, with outbreaks occurring in the U.S., Canada, and Europe.

With increased surveillance, *E. coli* O157:H7 has been recognized in other areas of the world including Mexico, China, Argentina, Belgium, and Thailand (N. V. Padhye and M. P. Doyle, "Escherichia coli O157:H7: Epidemiology, pathogenesis and methods for detection in food," *J. Food. Prot.*, 55: 555-565 [1992]; and P. M. Griffin and R. V. Tauxe, "The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome," *Epidemiol. Rev.*, 13: 60 [1991]).

The disease attracted national attention in the U.S. after a major outbreak in the Pacific Northwest that was associated with consumption of undercooked *E. coli* O157:H7-contaminated hamburgers. Over 700 hundred people fell ill (more than 170 were hospitalized) and four young children died (P. Recer, "Experts call for irradiation of meat to protect against food-borne bacteria," *Associated Press*, 7/12/94 [1994]). Several outbreaks since then have underscored the potential severity and multiple mechanisms for transmission of VTEC-associated diseases (M. Bielaszewska *et al.*, "Verotoxigenic (enterohaemorrhagic) *Escherichia coli* in infants and toddlers in Czechoslovakia," *Infection* 18: 352-356 [1990]; A. Caprioli *et al.*, "Hemolytic-uremic syndrome and Vero cytotoxin-producing *Escherichia coli* infection in Italy," *J. Infect. Dis.*, 166: 184-158 [1992]; A. Caprioli, *et al.*, "Community-wide Outbreak of Hemolytic-Uremic Syndrome Associated with Non-O157 Verocytotoxin-Producing *Escherichia coli*," *J. Infect. Dis.*, 169: 208-211 [1994]; N. Cimolai, "Low frequency of high level Shiga-like toxin production in enteropathogenic *Escherichia coli* serogroups," *Eur. J. Pediatr.*, 151: 147 [1992]; and R. Voelker, "Panel calls *E. coli* screening inadequate," *Escherichia coli* O157:H7--Panel sponsored by the American Gastroenterological Association Foundation in July 1994, *Medical News & Perspectives*, *J. Amer. Med. Assoc.*, 272: 501 [1994]).

While O157:H7 is currently the predominant *E. coli* serotype associated with illness in North America, other serotypes (as shown in Table 1, and in particular O26:H11, O113:H21, O91:H21 and O111:NM) also produce verotoxins which appear to be important in the pathogenesis of gastrointestinal manifestations and the hemolytic uremic syndrome (P. M. Griffin and R. V. Tauxe, "The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome," *Epidemiol. Rev.*, 13: 60 [1990]; M. M. Levine, *et al.*, "Antibodies to Shiga holotoxin and to two synthetic peptides of the B subunit in sera of patients with *Shigella dysenteriae* 1

dysentery." *J. Clin. Microbiol.*, 30: 1636-1641 [1992]; and C. R. Dorn, *et al.*, "Properties of Vero cytotoxin producing *Escherichia coli* of human and animal origin belonging to serotypes other than O157:H7," *Epidemiol. Infect.*, 103: 83-95 [1989]). Since organisms with these serotypes have been shown to cause illness in humans they may assume greater public health 5 importance over time (P. M. Griffin and R. V. Tauxe, "The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome," *Epidemiol. Rev.*, 13: 60 [1990]).

Clinicians usually observe cases of hemolytic uremic syndrome ("HUS") clustered in a geographic region. However, small outbreaks are likely to be missed because many 10 laboratories do not routinely screen stool specimens for *E. coli* O157:H7. Many cases related to non-commercial food preparation also probably go unrecognized. Nonetheless, *E. coli* O157:H7 is responsible for a large number of cases, as more than 20,000 cases of *E. coli* O157:H7 infection are reported annually in the U.S., with 400-500 deaths from HUS. 15 However, these estimates were compiled when only 11 states mandated reporting of *E. coli* O157:H7. Twenty-nine states have recently made *E. coli* O157:H7 infection a reportable disease (R. Voelker, "Panel calls *E. coli* screening inadequate: *Escherichia coli* O157:H7; panel sponsored by the American Gastroenterological Association Foundation in July 1994, Medical News & Perspectives," *J. Amer. Med. Assoc.*, 272: 501 [1994]). Indeed, the Centers for Disease Control recently added *E. coli* O157:H7 to their list of reportable diseases 20 ("Public Health Threats," *Science* 267:1427 [1995]).

C. Nature of Verotoxin-Induced Disease

Risk factors for HUS progression following infection with *E. coli* O157:H7 include age (very young or elderly), bloody diarrhea, leukocytosis, fever, large amounts of ingested 25 pathogen, previous gastrectomy, and the use of antimicrobial agents (in particular, trimethoprim-sulfamethoxazole)(A. A. Harris *et al.*, "Results of a screening method used in a 12 month stool survey for *Escherichia coli* O157:H7," *J. Infect. Dis.*, 152: 775-777 [1985]; and M. A. Karmali, "Infection by Verocytotoxin-producing *Escherichia coli*," *Clin. Microbiol. Rev.*, 2: 15-38 [1989]).

As indicated above, *E. coli* O157:H7 is associated with significant morbidity and 30 mortality. The spectrum of illness associated with *E. coli* O157:H7 infection includes asymptomatic infection, mild uncomplicated diarrhea, hemorrhagic colitis, HUS, and TTP". Hemorrhagic colitis (or "ischemic colitis") is a distinct clinical syndrome characterized by

sudden onset of abdominal cramps—likened to the pain associated with labor or appendicitis—followed within 24 hours by watery diarrhea. One to two days later, the diarrhea turns grossly bloody in approximately 90% of patients and has been described as "all blood and no stool" (C. H. Pai *et al.*, "Sporadic cases of hemorrhagic colitis associated with 5 *Escherichia coli* O157:H7," Ann. Intern. Med., 101: 738-742 [1984]; and R. S. Remis *et al.*, "Sporadic cases of hemorrhagic colitis associated with *Escherichia coli* O157:H7," Ann. Intern. Med., 101: 738-742 [1984]). Vomiting may occur, but there is little or no fever. The time from ingestion to first loose stool ranges from 3–9 days (with a mean of 4 days) L. W. Riley *et al.*, "Hemorrhagic colitis associated with a rare *Escherichia coli* serotype," New Eng. 10 J. Med., 308: 681-685 [1983]; and D. Pudden *et al.*, "Hemorrhagic colitis in a nursing home," Ontario Can. Dis. Weekly Rpt., 11: 169-170 [1985]), and the duration of illness ranges generally from 2–9 days (with a mean of 4 days).

HUS is a life-threatening blood disorder that appears within 3–7 days following onset 15 of diarrhea in 10–15% of patients. Those younger than 10 years and the elderly are at particular risk. Symptoms include renal glomerular damage, hemolytic anemia (rupturing of erythrocytes as they pass through damaged renal glomeruli), thrombocytopenia and acute kidney failure. Approximately 15% of patients with HUS die or suffer chronic renal failure. Indeed, HUS is a leading cause of renal failure in childhood (reviewed by M.A. Karmali, "Infection by Verocytotoxin-producing *Escherichia coli*," Clin. Microbiol. Rev., 2: 15-38 20 [1989]). Currently, blood transfusion and dialysis are the only therapies for HUS.

TTP shares similar histopathologic findings with HUS, but usually results in multiorgan microvascular thrombosis. Neurological signs and fever are more prominent in 25 TTP, compared with HUS. Generally occurring in adults, TTP is characterized by microangiopathic hemolytic anemia, profound thrombocytopenia, fluctuating neurologic signs, fever and mild azotemia (H. C. Kwaan, "Clinicopathological features of thrombotic thrombocytopenic purpura," Semin. Hematol., 24: 71-81 [1987]; and S. J. Machin, "Clinical annotation: Thrombotic thrombocytopenic purpura," Br. J. Hematol., 56: 191-197 [1984]). Patients often die from microthrombi in the brain. In one review of 271 cases, a rapidly progressive course was noted, with 75% of patients dying within 90 days (E.L. Amorosi and 30 J.E. Ultmann, "Thrombotic thrombocytopenic purpura: Report of 16 cases and review of the literature," Med., 45:139-159 (1966)).

Other diseases associated with *E. coli* O157:H7 infection include hemorrhagic cystitis and balantitis (W. R. Grandsen *et al.*, "Hemorrhagic cystitis and balantitis associated with

verotoxin-producing *Escherichia coli* O157:H7." *Lancet* ii: 150 [1985]). convulsions, sepsis with other organisms and anemia (P. C. Rowe *et al.*, "Hemolytic anemia after childhood *Escherichia coli* O157:H7 infection: Are females at increased risk?" *Epidemiol. Infect.* 106: 523-530 [1991]).

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D. Mechanism of Pathogenesis

Verotoxins are strongly linked to *E. coli* O157:H7 pathogenesis. All clinical isolates of *E. coli* O157:H7 have been shown to produce one or both verotoxins (VT1 and VT2) (C. A. Bopp *et al.*, "Unusual Verotoxin-producing *Escherichia coli* associated with hemorrhagic colitis." *J. Clin. Microbiol.* 25: 1486-1489 [1987]). Both of these toxins are cytotoxic to Vero (African green monkey kidney) and HeLa cells. and cause paralysis and death in mice (A. D. O'Brien *et al.*, "Purification of *Shigella dysenteriae* 1 (Shiga) like toxin from *Escherichia coli* O157:H7 strain associated with hemorrhagic colitis." *Lancet* ii: 573 [1983]). These toxins are sometimes referred to in the literature as Shiga-like toxins I and II (SLT-I and SLT-II, respectively), due to their similarities with the toxins produced by *Shigella*. Indeed, much of our understanding of *E. coli* VTs is based on information accumulated on Shiga toxins. Shiga toxin, first described in 1903, has been recognized as one of the most potent bacterial toxins for eukaryotic cells (reviewed by M.A. Karmali, "Infection by Verocytotoxin-producing *Escherichia coli*," *Clin. Microbiol. Rev.*, 2: 15-38 [1989]).

20 Hereinafter, the VT convention will be used; thus, VT1 and VT2 correspond to SLT-I and SLT-II, respectively.

While the pathogenic mechanism of *E. coli* O157:H7 infection is incompletely understood, it is believed that ingested organisms adhere to and colonize the intestinal mucosa, where toxins are released which cause endothelial cell damage and bloody diarrhea. It is also postulated that hemorrhagic colitis progresses to HUS when verotoxins enter the bloodstream, damaging the endothelial cells of the microvasculature and triggering a cascade of events resulting in thrombus deposition in small vessels. These microthrombi occlude the microcapillaries of the kidneys (particularly in the glomeruli) and other organs, resulting in their failure (J. J. Byrnes and J. L. Moake, "TTP and HUS syndrome: Evolving concepts of pathogenesis and therapy," *Clin. Hematol.*, 15: 413-442 [1986]; and T. G. Cleary, "Cytotoxin-producing *Escherichia coli* and the hemolytic uremic syndrome," *Pediatr. Clin. North Am.*, 35: 485-501 [1988]). Verotoxins entering the bloodstream may also result in direct kidney cytotoxicity.

VT1 is immunologically and structurally indistinguishable from Shiga toxin produced by *Shigella dysenteriae* (A. D. O'Brien *et al.*, "Purification of *Shigella dysenteriae* 1 (Shiga) like toxin from *Escherichia coli* O157:H7 strain associated with hemorrhagic colitis," *Lancet* ii: 573 [1983]). VT1 and VT2 holotoxins each consist of one A and five B subunits (A. Donohue-Rolfe *et al.*, "Purification of Shiga toxin and Shiga-like toxins I and II by receptor analog affinity chromatography with immobilized P1 glycoprotein and production of cross reactive monoclonal antibodies," *Infect. Immun.*, 57: 3888-3893 [1989]; and A. Donohue-Rolfe *et al.*, "Simplified high yield purification of *Shigella* toxin and characterization of subunit composition and function by the use of subunit-specific monoclonal and polyclonal antibodies," *J. Exp. Med.*, 160: 1767-1781 [1984]). The toxic A subunit is enzymatically active, while the B subunit binds the holotoxin to the receptor on the target eukaryotic cell.

Crystal structure analysis of Shiga holotoxin and VT1 B subunit pentamers have shown that the holotoxin assembles with the C-terminal end of the A subunit associating with, and inserting within, a pentamer of B chains (P. E. Stein *et al.*, "Crystal structure of the cell-binding B oligomer of verotoxin-1 from *E. coli*," *Nature* 355: 748-750 [1992]; and M.E. Fraser *et al.*, "Crystal structure of the holotoxin from *Shigella dysenteriae* at 2.5 Å resolution," *Struct. Biol.*, 1:59-64 [1994]). This conformation is consistent with the observation that a C-terminally truncated A1 subunit of VT1 is toxic (in a ribosomal inhibition assay), but cannot associate with B subunit pentamers (P. R. Austin *et al.*, "Evidence that the A₁ fragment of Shiga-like toxin type I is required for holotoxin integrity," *Infect. Immun.*, 62: 1768 [1994]).

The Verotoxin A Subunit. Examination of the crystal structure of Shiga holotoxin indicates that the N-terminus of its A subunit is both surface-exposed and functionally important. Removal of amino acid interval 3-18 of the A subunit completely abolished toxicity (L. P. Perera *et al.*, "Mapping the minimal contiguous gene segment that encodes functionally active Shiga-like toxin II," *Infect. Immun.*, 59: 829-835 [1991]) while removal of interval 25-44 retained toxicity but abolished its association with B subunit pentamers (J. E. Haddad *et al.*, "Minimum domain of the Shiga toxin A subunit required for enzymatic activity," *J. Bacteriol.*, 175: 4970-4978 [1993]). Deletion of the first 13 residues of the homologous ricin A subunit also abolished toxicity, while deletion of the first 9 residues did not (M. J. May, *et al.*, "Ribosome inactivation by ricin A chain: A sensitive method to assess the activity of wild-type and mutant polypeptides," *EMBO J.*, 8: 301-308 [1989]).

The Verotoxin B Subunit. Studies of Shiga toxin B subunit suggest that neutralizing epitopes may also be present at both the N- and C-terminal regions of VT1 and VT2 B

subunits. Polyclonal antibodies raised against peptides from these regions (residues 5-18, 13-26, 7-26, 54-67 and 57-67) show partial neutralization of Shiga toxin (I. Harari and R. Arnon, "Carboxy-terminal peptides from the B subunit of Shiga toxin induce a local and parenteral protective effect," *Mol. Immunol.*, 27: 613-621 [1990]; and I. Harari *et al.*, "Synthetic peptides of Shiga toxin B subunit induce antibodies which neutralize its biological activity," *Infect. Immun.*, 56: 1618-1624 [1988]). Deletion of the last five amino acids of Shiga toxin B (M. P. Jackson *et al.*, "Functional Analysis of the Shiga toxin and Shiga-like toxin Type II variant binding subunits by using site-directed mutagenesis," *J. Bacteriol.*, 172: 653-658 [1990]), or four amino acids of VT2 B (L. P. Perera *et al.*, "Mapping the minimal contiguous gene segment that encodes functionally active Shiga-like toxin II," *Infect. Immun.*, 59: 829-835 [1991]), eliminate toxin activity, while deletion of the last two amino acids of VT2 B subunit reduced cytotoxicity. In contrast, the addition of an 18 or 21 amino acid extension to the native C-terminus of the VT2 B subunit was presumably conformationally correct, as these proteins assembled cytotoxic holotoxin.

Various approaches to express recombinant verotoxins have included individual or coordinate expression of A and B subunits from high-copy number plasmids and expression with fusion partners (J. E. Haddad *et al.*, "Minimum domain of the Shiga toxin A subunit required for enzymatic activity," *J. Bacteriol.*, 175: 4970-4978; J. E. Haddad and M. P. Jackson, "Identification of the Shiga toxin A-subunit residues required for holotoxin assembly," *J. Bacteriol.*, 175: 7652-7657 [1993]; M. P. Jackson *et al.*, "Mutational analysis of the Shiga toxin and Shiga-like toxin II enzymatic subunits," *J. Bacteriol.*, 172: 3346-3350 [1990]; C. J. Hovde *et al.*, "Evidence that glutamic acid 167 is an active-site residue of Shiga-like toxin I," *Proc. Natl. Acad. Sci.*, 85: 2568-2572 [1988]; R. L. Deresiewicz *et al.*, "The role of tyrosine-114 in the enzymatic activity of the Shiga-like toxin I A-chain," *Mol. Gen. Genet.*, 241: 467-473 [1993]; T. M. Zollman *et al.*, "Purification of Recombinant Shiga-like Toxin Type I A, Fragment from *Escherichia coli*," *Protein Express Purific.*, 5: 291-295 [1994]; K. Ramotar, *et al.*, "Characterization of Shiga-like toxin I B subunit purified from overproducing clones of the SLT-I B cistron," *Biochem J.*, 272: 805-811 [1990]; S. B. Calderwood *et al.*, "A system for production and rapid purification of large amounts of the Shiga toxin/Shiga-like toxin I B subunit," *Infect. Immun.*, 58: 2977-2982 [1990]; D. W. K. Acheson, *et al.*, "Comparison of Shiga-like toxin I B-subunit expression and localization in *Escherichia coli* and *Vibrio cholerae* by using *trc* or Iron-regulated promoter systems," *Infect. Immun.*, 61: 1098-1104 [1993]; M. P. Jackson *et al.*, "Nucleotide sequence analysis and

comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from *Escherichia coli* 933," FEMS Microbiol. Lett., 44: 109-114 [1987]; J. W. Newland *et al.*, "Cloning of genes for production of *Escherichia coli* Shiga-like toxin type II," Infect. Immun. 55: 2675-2680 [1987]; and F. Gunzer and H. Karch, "Expression of A and B subunits of Shiga-like toxin II as fusions with glutathione S-transferase and their potential for use in seroepidemiology.", J. Clin. Microbiol., 31: 2604-2610 [1993]; and D.W. Acheson *et al.*, "Expression and purification of Shiga-like toxin II B subunits," Inf. Immun., 63:301-308 [1995]). In one case, bench top fermentation techniques yielded 22 mg/liter of soluble recombinant protein (D. W. K. Acheson, *et al.*, "Comparison of Shiga-like toxin I B-subunit expression and localization in *Escherichia coli* and *Vibrio cholerae* by using *trc* or Iron-regulated promoter systems," Infect. Immun. 61: 1098-1104 [1993]). However, there have been no systematic approaches to identifying the appropriate spectrum of VT antigens, preserving immunogen and immunoabsorbant antigenicity and scaling-up.

The receptor for VT1 and VT2 is a globotriaosyl ceramide containing a galactose α -(1-4)- galactose- β -(1-4) glucose ceramide (Gb3) (C. A. Lingwood *et al.*, "Glycolipid binding of natural and recombinant *Escherichia coli* produced verotoxin *in vitro*," J. Biol. Chem., 262: 1779-1785 [1987]; and T. Wadell *et al.*, "Globotriaosyl ceramide is specifically recognized by the *Escherichia coli* verocytotoxin 2," Biochem. Biophys. Res. Commun., 152: 674-679 [1987]). Gb3 is abundant in the cortex of the human kidney and is present in primary human endothelial cell cultures. Hence, the identification of Gb3 as the functional receptor for VT1 and VT2 is consistent with their role in HUS pathogenesis, in which endothelial cells of the renal vasculature are the principal site of damage. Therefore, toxin-mediated pathogenesis may follow a sequence of B subunit binding to Gb3 receptors on kidney cells, toxin internalization, enzymatic reduction of the A subunit to an A1 fragment, binding of the A1 subunit to the 60S ribosomal subunit, inhibition of protein synthesis and cell death (A. D. O'Brien *et al.*, "Shiga and Shiga-like toxins. Microbial Rev., 51: 206-220 [1987]).

The role of verotoxins in the pathogenesis of *E. coli* O157:H7 infections has been further studied in animal models. Infection or toxin challenge of laboratory animals do not produce all the pathologies and symptoms of hemorrhagic colitis, HUS, and TTP which occur in humans. Glomerular damage is noticeably absent. Nonetheless, experiments using animal models implicate verotoxins as the direct cause of hemorrhagic colitis, microvascular damage leading to the failure of kidneys and other organs and CNS neuropathies.

For example, Barrett, *et al.* delivered VT2 into the peritoneal cavity of rabbits using mini-osmotic pumps (J. J. Barrett *et al.*, "Continuous peritoneal infusion of shiga-like toxin II (SLTII) as a model for SLT II-induced diseases," *J. Infect. Dis.* 159: 774-777 [1989]). In three days, most animals receiving the toxin developed diarrhea, with intestinal lesions resembling those seen in humans with hemorrhagic colitis. Although there was some evidence of renal dysfunction, none of the rabbits developed HUS. Beery, *et al.* showed that VT2, when administered intraperitoneally or intravenously to adult mice, produces lesions of the kidneys and colon (J. T. Beery *et al.*, "Cytotoxic activity of *Escherichia coli* O157:H7 culture filtrate on the mouse colon and kidney," *Curr. Microbiol.* 11: 335-342 [1984]).

10 Histologic lesions in the kidney included accumulation of numerous exfoliated collecting tubules and marked intracellular vacuolation of proximal convoluted tubular cells.

15 Sjögren *et. al.* studied the pathogenesis of an entero-adherent strain of *E. coli* (RDEC-1) lysogenized with a VT1-containing bacteriophage (VT1-producing RDEC-1) (R. Sjögren *et al.*, "Role of Shiga-like toxin I in bacterial enteritis: comparison between isogenic *Escherichia coli* strains induced in rabbits," *Gastroenterol.*, 106: 306-317 [1994]). In this study, rabbits were challenged with RDEC-1 or VT1-producing RDEC-1 and studied for onset of disease. The VT1-producing variant induced a severe, non-invasive, entero-adherent infection in rabbits which was characterized by serious histological lesions with vascular changes, edema and severe epithelial inflammation. Importantly, vascular changes consistent with endothelial damage were seen in infected animals that was similar to intestinal microvascular changes in humans with *E. coli* O157:H7 infection. Based on these observations, they concluded that VT1 is an important virulence factor in enterohemorrhagic *E. coli* O157:H7 infection.

20 Fuji *et. al.* described a model in which mice were treated for three days with streptomycin followed by a simultaneous challenge of *E. coli* O157:H7 orally, and mitomycin intraperitoneally (J. Fuji *et al.*, "Direct evidence of neuron impairment by oral infection with Verotoxin-producing *Escherichia coli* O157:H7 in mitomycin-treated mice," *Infect. Immun.* 62: 3447-34453 [1994]). All of the animals died within four days. Immunoelectron-microscopy strongly suggested that death was due to the toxic effects of VT2v (a structural variant of VT2), on both the endothelial cells and neurons in the central nervous system which resulted in fatal acute encephalopathy.

25 30 Wadolkowski *et al.* studied colonization of *E. coli* O157:H7 in mice. Mice were treated with streptomycin and fed 10^{10} *E. coli* O157:H7 (E. A. Wadolkowski *et al.*, "Mouse

model for colonization and disease caused by enterohemorrhagic *Escherichia coli* O157:H7." Infect. Immun. 58: 2438-2445 [1990]; and E. A. Wadolkowski *et al.*, "Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shiga-like toxin Type II." Infect. Immun. 58: 3959-3965 [1990]). All of the mice died due to 5 severe, disseminated, acute necrosis of proximal convoluted tubules. In mouse models, glomerular damage was not observed, but toxic acute renal tubular necrosis was observed which is characteristic of some HUS patients. The failure of mice to show glomerular damage is thought to be due to the absence of a functional globotriaosyl ceramide receptor specific for verotoxins in the glomeruli of the kidneys. Administration of VT2 subunit- 10 specific monoclonal antibodies prior to infection prevented all pathology and death.

E. Current Therapeutic Approaches

E. coli O157:H7 disease is not adequately controlled by current therapy. Patient treatment is tailored to manage fluid and electrolyte disturbances, anemia, renal failure and hypertension. Although *E. coli* O157:H7 is susceptible to common antibiotics, the role of 15 antibiotics in the treatment of infection has questionable merit. In both retrospective and prospective studies, prophylaxis or treatment with antibiotics such as trimethoprim-sulfamethoxazole, there was either no benefit or an increased risk of developing HUS (T. N. Bokete *et al.*, "Shiga-like toxin producing *Escherichia coli* in Seattle children: a prospective study." Gastroenterol. 105: 1724-1731 [1993]; A. T. Pavia *et al.*, "Hemolytic uremic- 20 syndrome during an outbreak of *Escherichia coli* O157:H7 infections in institutions for mentally retarded persons: clinical and epidemiologic observations." J. Pedatr. 116: 544-551 [1990]; F. Proulx *et al.*, "Randomized, controlled trial of antibiotic therapy for *Escherichia coli* O157:H7 enteritis." J. Pediatr. 121: 299-303 [1992]; and A. L. Carter *et al.*, "A severe 25 outbreak of *Escherichia coli* O157:H7-associated hemorrhagic colitis in a nursing home." New Eng. J. Med. 317: 1496-1500 [1987]).

The mechanisms by which antibiotics increase the risk of infection or related complications might involve enhancement of toxin production, release of toxins from killed organisms, or alteration of normal competing intestinal flora allowing for pathogen overgrowth (M. A. Karmali, "Infection by Verocytotoxin-producing *Escherichia coli*." Clin. 30 Microbiol. Rev. 2: 15-38 [1989]). A further concern in the use of antibiotics is the potential acquisition of antimicrobial resistance by *E. coli* O157:H7 (C. R. Dorn, "Review of foodborne outbreak of *Escherichia coli* O157:H7 infection in the western United States." JAVMA 203: 1583-1587 [1993]).

In addition, by the time symptoms are serious enough to attract medical attention, it is likely that verotoxins are already entering the systemic circulation or will do so shortly thereafter. Although antimicrobials may help to prevent pathology resulting from the action of toxin on the bowel lumen. However, by the time symptoms of HUS have developed, the 5 patient has ceased shedding organisms. Thus, antimicrobial treatment during HUS disease is of less value, and often contraindicated, due to the increased risk of complications associated with administration of antimicrobials to patients susceptible to development of HUS. Importantly, there is currently no antitoxin commercially available for use in treating affected 10 patients. What is needed is a means to block the progression of disease, without the complications associated with antimicrobial treatment.

DESCRIPTION OF THE DRAWINGS

Figure 1 is an SDS-PAGE of rVT1 and rVT2.

Figure 2 shows HPLC results for rVT1 and rVT2.

15 Figure 3 shows rVT1 and rVT2 toxicity in Vero cell culture.

Figure 4 shows EIA reactivity of rVT1 and rVT2 antibodies to rVT1.

Figure 5 shows EIA reactivity of rVT1 and rVT2 Antibodies to rVT2.

Figure 6 shows Western Blot reactivity of rVT1 and rVT2 antibodies to rVT's:

20 Panel 6A contains preimmune IgY;

Panel 6B contains rVT1 IgY; and

Panel 6C contains rVT2 IgY.

Figure 7 shows neutralization of rVT1 cytotoxicity in Vero cells.

Figure 8 shows neutralization of rVT2 cytotoxicity in Vero cells.

Figure 9 shows renal sections from *E. coli* O157:H7-infected mice treated with IgY

25 Panel 9A shows a representative kidney section from a mouse treated with preimmune IgY;

Panel 9B shows a representative kidney sections from a mouse treated with rVT1; and

Panel 9C shows a representative kidney section from a mouse treated with rVT2 IgY.

30 Figure 10 shows the fusion constructs of VT components and affinity tags.

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

As used herein, the term "neutralizing" is used in reference to antitoxins, particularly antitoxins comprising antibodies, which have the ability to prevent the pathological actions of the toxin against which the antitoxin is directed.

As used herein, the term "overproducing" is used in reference to the production of toxin polypeptides in a host cell, and indicates that the host cell is producing more of the toxin by virtue of the introduction of nucleic acid sequences encoding the toxin polypeptide than would be expressed by the host cell absent the introduction of these nucleic acid sequences. To allow ease of purification of toxin polypeptides produced in a host cell it is preferred that the host cell express or overproduce the toxin polypeptide at a level greater than 1 mg/liter of host cell culture.

As used herein, the term "fusion protein" refers to a chimeric protein containing the protein of interest (*i.e.*, an *E. coli* verotoxin and/or fragments thereof) joined to an exogenous protein fragment (the fusion partner which consists of a non-toxin protein). The fusion partner may enhance solubility of the *E. coli* protein as expressed in a host cell, may provide an "affinity tag" to allow purification of the recombinant fusion protein from the host cell or culture supernatant, or both. If desired, the fusion protein may be removed from the protein of interest (*i.e.*, toxin protein or fragments thereof) prior to immunization by a variety of enzymatic or chemical means known to the art.

As used herein, the term "affinity tag" refers to such structures as a "poly-histidine tract" or "poly-histidine tag," or any other structure or compound which facilitates the purification of a recombinant fusion protein from a host cell, host cell culture supernatant, or both. As used herein, the term "flag tag" refers to short polypeptide marker sequence useful for recombinant protein identification and purification.

As used herein, the terms "poly-histidine tract" and "poly-histidine tag," when used in reference to a fusion protein refers to the presence of two to ten histidine residues at either the amino- or carboxy-terminus of a protein of interest. A poly-histidine tract of six to ten residues is preferred. The poly-histidine tract is also defined functionally as being a number of consecutive histidine residues added to the protein of interest which allows the affinity purification of the resulting fusion protein on a nickel-chelate column.

As used herein, the term "chimeric protein" refers to two or more coding sequences obtained from different genes, that have been cloned together and that, after translation, act as

a single polypeptide sequence. Chimeric proteins are also referred to as "hybrid proteins." As used herein, the term "chimeric protein" refers to coding sequences that are obtained from different species of organisms, as well as coding sequences that are obtained from the same species of organisms.

5 As used herein, the term "protein of interest" refers to the protein whose expression is desired within the fusion protein. In a fusion protein, the protein of interest will be joined or fused with another protein or protein domain, the fusion partner, to allow for enhanced stability of the protein of interest and/or ease of purification of the fusion protein.

As used herein, the term "maltose binding protein" and "MBP" refers to the maltose binding protein of *E. coli*. A portion of the maltose binding protein may be added to a protein of interest to generate a fusion protein; a portion of the maltose binding protein may merely enhance the solubility of the resulting fusion protein when expressed in a bacterial host. On the other hand, a portion of the maltose binding protein may allow affinity purification of the fusion protein on an amylose resin.

10 As used herein, the term "purified" or "to purify" refers to the removal of contaminants from a sample. For example, antitoxins are purified by removal of contaminating non-immunoglobulin proteins; they are also purified by the removal of substantially all immunoglobulin that does not bind toxin. The removal of non-immunoglobulin proteins and/or the removal of immunoglobulins that do not bind toxin results in an increase in the percent of toxin-reactive immunoglobulins in the sample. In another example, recombinant toxin polypeptides are expressed in bacterial host cells and the toxin polypeptides are purified by the removal of host cell proteins: the percent of recombinant toxin polypeptides is thereby increased in the sample.

15 The term "recombinant DNA molecule" as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques.

The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed from a recombinant DNA molecule.

20 The term "native protein" as used herein refers to a protein which is isolated from a natural source as opposed to the production of a protein by recombinant means.

25 As used herein the term "portion" when in reference to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid.

As used herein "soluble" when in reference to a protein produced by recombinant DNA technology in a host cell, is a protein which exists in solution in the cytoplasm of the host cell; if the protein contains a signal sequence, the soluble protein is exported to the periplasmic space in bacterial hosts and is secreted into the culture medium of eukaryotic cells capable of secretion or by bacterial hosts possessing the appropriate genes. In contrast, an insoluble protein is one which exists in denatured form inside cytoplasmic granules (called an inclusion bodies) in the host cell. High level expression (*i.e.*, greater than 1 mg recombinant protein/liter of bacterial culture) of recombinant proteins often results in the expressed protein being found in inclusion bodies in the bacterial host cells. A soluble protein is a protein which is not found in an inclusion body inside the host cell or is found both in the cytoplasm and in inclusion bodies and in this case the protein may be present at high or low levels in the cytoplasm.

A distinction is drawn between a soluble protein (*i.e.*, a protein which when expressed in a host cell is produced in a soluble form) and a "solubilized" protein. An insoluble recombinant protein found inside an inclusion body may be solubilized (*i.e.*, rendered into a soluble form) by treating purified inclusion bodies with denaturants such as guanidine hydrochloride, urea or sodium dodecyl sulfate (SDS). These denaturants must then be removed from the solubilized protein preparation to allow the recovered protein to renature (refold). Not all proteins will refold into an active conformation after solubilization in a denaturant and removal of the denaturant. Many proteins precipitate upon removal of the denaturant. SDS may be used to solubilize inclusion bodies and will maintain the proteins in solution at low concentration. However, dialysis will not always remove all of the SDS (SDS can form micelles which do not dialyze out); therefore, SDS-solubilized inclusion body protein is soluble but not refolded.

As used herein, the term "reporter reagent" or "reporter molecule" is used in reference to compounds which are capable of detecting the presence of antibody bound to antigen. For example, a reporter reagent may be a colorimetric substance which is attached to an enzymatic substrate. Upon binding of antibody and antigen, the enzyme acts on its substrate and causes the production of a color. Other reporter reagents include, but are not limited to fluorogenic and radioactive compounds or molecules.

As used herein the term "signal" is used in reference to the production of a sign that a reaction has occurred, for example, binding of antibody to antigen. It is contemplated that signals in the form of radioactivity, fluorogenic reactions, and enzymatic reactions will be

used with the present invention. The signal may be assessed quantitatively as well as qualitatively.

As used herein, the term "therapeutic amount" refers to that amount of antitoxin required to neutralize the pathologic effects of *E. coli* toxin in a subject.

5 As used herein, the term "acute intoxication" is used in reference to cases of *E. coli* infection in which the patient is currently suffering from the effects of toxin (e.g., *E. coli* verotoxins or enterotoxins). Signs and symptoms of intoxication with the toxin may be immediately apparent. Or, the determination of intoxication may require additional testing, such as detection of toxin present in the patient's fecal material.

10 As used herein, the term "at risk" is used in references to individuals who have been exposed to *E. coli* and may suffer the symptoms associated with infection or disease with these organisms, especially due to the effects of verotoxins.

SUMMARY OF THE INVENTION

15 The present invention relates to antitoxin therapy for humans and other animals. Antitoxins which neutralize the pathologic effects of *E. coli* toxins are generated by immunization of avian hosts with recombinant toxin fragments. In one embodiment, the present invention contemplates a method of treatment administering at least one antitoxin directed against at least a portion of an *Escherichia coli* verotoxin in an aqueous solution in 20 therapeutic amount that is administrable to an intoxicated subject. It is contemplated that the intoxicated subject will be either an adult or a child.

25 In a preferred embodiment, the *E. coli* verotoxin is recombinant. In one embodiment, the antitoxin is an avian antitoxin. In an alternative embodiment, the recombinant *E. coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT1 sequence. In one embodiment of the *E. coli* fusion protein, the fusion protein comprises a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT2 sequence.

30 Various routes of administration, are contemplated for providing the *E. coli* antitoxin(s) to an affected individual, including but not limited to, parenteral as well as oral routes of administration. In a particularly preferred embodiment, the route of administration is parenteral.

The present invention also includes the embodiment of a method of prophylactic treatment in which an antitoxin directed against at least one *E. coli* verotoxin in an aqueous

solution in therapeutic amount that is parenterally administrable, and is administered to at least one subject at risk of diarrheal disease. In one embodiment, the antitoxin is parenterally administered.

5 In one embodiment, the subject is at risk of developing extra-intestinal complications of *E. coli* infections, including but not limited to, hemolytic uremic syndrome, thrombotic thrombocytopenic purpura, etc.

The present invention also includes the embodiment of a composition which comprises neutralizing antitoxin directed against at least one *E. coli* verotoxin in an aqueous solution in therapeutic amounts. In one particularly preferred embodiment, the *E. coli* verotoxin is a recombinant toxin. In an alternative embodiment, the recombinant *E. coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *E. coli* verotoxin VT1 sequence. In another embodiment, the recombinant *E. coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *E. coli* verotoxin VT2 sequence. In yet another embodiment, the composition of the antitoxin is directed against a portion of at least one *Escherichia coli* verotoxin. In one embodiment, the portion of *Escherichia coli* is selected from the group consisting of subunit A and subunit B of VT1. In an alternative embodiment, the portion of *Escherichia coli* is selected from the group consisting of subunit A and subunit B of VT2. Indeed, the invention contemplates an antitoxin that is directed against a portion of at least one *Escherichia coli* verotoxin. In one embodiment, the antitoxin is an avian antitoxin.

The present invention also comprises a method of treatment of enteric bacterial infections comprising administering an avian antitoxin directed against at least one verotoxin produced by *E. coli* in an aqueous solution in therapeutic amount, to at least one infected subject. In one preferred embodiment, the avian antitoxin is administered parenterally.

25 In another embodiment, the *E. coli* is selected from the group consisting of *Escherichia coli* serotypes O157:H7, O1:NM; O2:H5; O2:H7; O4:NM; O4:H10; O5:NM; O5:H16; O6:H1; O18:NM; O18:H7; O25:NM; O26:NM; O26:H11; O26:H32; O38:H21; O39:H4; O45:H2; O50:H7; O55:H7; O55:H10; O82:H8; O84:H2; O91:NM; O91:H21; O103:H2; O111:NM; O111:H8; O111:H30; O111:H34; O113:H7; O113:H21; O114:H48; O115:H10; O117:H4; O118:H12; O118:H30; O121:NM; O121:H19; O125:NM; O125:H8; O126:NM; O126:H8; O128:NM; O128:H2; O128:H8; O128:H12; O128:H25; O145:NM; O125:H25; O146:H21; O153:H25; O157:NM; O163:H19; O165:NM; O165:19; and O165:H25. In one embodiment, the antitoxin comprises antitoxin directed against at least one

Escherichia coli verotoxin. In another embodiment, the antitoxin is cross-reacts with at least one *Escherichia coli* verotoxin. In yet another embodiment, the antitoxin is reactive against toxins produced by members of the genus *Shigella*, including *S. dysenteriae*.

The present invention also contemplates uses for the toxin fragments in vaccines and diagnostic assays. The fragments may be used separately as purified, soluble antigens or, alternatively, in mixtures or "cocktails." The present invention thus comprises a method for detecting *Escherichia coli* verotoxin in a sample in which a sample, an antitoxin raised against *Escherichia coli* verotoxin, and a reporter reagent capable of binding the antitoxin are provided. The antitoxin is added to the sample, so that the antitoxin binds to the *E. coli* verotoxin in the sample. In one embodiment, the antitoxin is an avian antitoxin. In an alternative embodiment, the method further comprises the steps of washing unbound antitoxin from the sample, adding at least one reporter reagent to the sample, so that said reporter reagent binds to any antitoxin that is bound, washing the unbound reporter reagent from the sample and detecting the reporter reagent bound to the antitoxin bound to the *Escherichia coli* verotoxin, so that the verotoxin is detected. In one embodiment, the detecting is accomplished through any means, such as enzyme immunoassay, radioimmunoassay, fluorescence immunoassay, flocculation, particle agglutination, and *in situ* chromogenic assay. In one preferred embodiment, the sample is a biological sample. In an alternative preferred embodiment, the sample is an environmental sample.

20

DESCRIPTION OF THE INVENTION

The present invention contemplates treating humans and other animals intoxicated with at least one bacterial toxin. It is contemplated that administration of antitoxin will be used to treat patients effected by or at risk of symptoms due to the action of bacterial toxins. It is also contemplated that the antitoxin will be used in a diagnostic assay to detect the presence of toxins in samples. The organisms, toxins and individual steps of the present invention are described separately below.

I. Antibodies Directed Against *E. coli* and Associated Toxins

30

A preferred embodiment of the method of the present invention is directed toward obtaining antibodies against various *E. coli* serotypes, their toxins, enzymes or other metabolic by-products, cell wall components, or synthetic or recombinant versions of any of these compounds. It is contemplated that these antibodies will be produced by immunization

of humans or other animals. It is not intended that the present invention be limited to any particular toxin or any species of organism. In one embodiment, toxins from all *E. coli* serotypes are contemplated as immunogens. Examples of these toxins include the verotoxins VT1 and VT2.

5 It is not intended that antibodies produced against one toxin will only be used against that toxin. It is contemplated that antibodies directed against one toxin may be used as an effective therapeutic against one or more toxin(s) produced by other *E. coli* serotypes, or other toxin producing organisms (e.g., *Shigella*, *Bacillus cereus*, *Staphylococcus aureus*, *Streptococcus mutans*, *Acinetobacter calcoaceticus*, *Pseudomonas aeruginosa*, other 10 *Pseudomonas* species, *Vibrio* species, *Clostridium* species, etc.). It is further contemplated that antibodies directed against the portion of the toxin which binds to mammalian membranes can also be used against other organisms. It is contemplated that these membrane binding domains are produced synthetically and used as immunogens.

15 **II. Obtaining Antibodies In Non-Mammals**

A preferred embodiment of the method of the present invention for obtaining antibodies involves immunization. However, it is also contemplated that antibodies may be obtained from non-mammals without immunization. In the case where no immunization is contemplated, the present invention may use non-mammals with preexisting antibodies to 20 toxins as well as non-mammals that have antibodies to whole organisms by virtue of reactions with the administered antigen. An example of the latter involves immunization with synthetic peptides or recombinant proteins sharing epitopes with whole organism components.

In a preferred embodiment, the method of the present invention contemplates 25 immunizing non-mammals with bacterial toxin(s). It is not intended that the present invention be limited to any particular toxin. In one embodiment, toxins from all *E. coli* serotypes are contemplated as immunogens.

A particularly preferred embodiment involves the use of bacterial toxin protein or 30 fragments of toxin proteins produced by molecular biological means (i.e., recombinant toxin proteins). In a preferred embodiment, the immunogen comprises recombinant VT1 and/or VT2.

When immunization is used, the preferred non-mammal is from the class *Aves*. All birds are contemplated (e.g., duck, ostrich, emu, turkey, etc.). A preferred bird is a chicken. Importantly, chicken antibody does not fix mammalian complement (See H.N. Benson *et al.*,

J. Immunol. 87:616 [1961]). Thus, chicken antibody will normally not cause a complement-dependent reaction (A.A. Benedict and K. Yamaga. *"Immunoglobulins and Antibody Production in Avian Species,"* in *Comparative Immunology* (J.J. Marchaloni, ed.), pp. 335-375, Blackwell, Oxford [1966]). Thus, the preferred antitoxins of the present invention will 5 not exhibit complement-related side effects observed with antitoxins presently known.

When birds are used, it is contemplated that the antibody will be obtained from either the bird serum or the egg. A preferred embodiment involves collection of the antibody from the egg. Laying hens transport immunoglobulin to the egg yolk ("IgY") in concentrations equal to or exceeding that found in serum (See R. Patterson *et al.*, J. Immunol. 89:272 10 (1962); and S.B. Carroll and B.D. Stollar, J. Biol. Chem. 258:24 [1983]). In addition, the large volume of egg yolk produced vastly exceeds the volume of serum that can be safely obtained from the bird over any given time period. Finally, the antibody from eggs is more pure and more homogeneous: there is far less non-immunoglobulin protein (as compared to serum) and only one class of immunoglobulin is transported to the yolk.

15 When considering immunization with toxins, one may consider modification of the toxins to reduce the toxicity. In this regard, it is not intended that the present invention be limited by immunization with modified toxin. Unmodified ("native") toxin is also contemplated as an immunogen.

It is also not intended that the present invention be limited by the type of modification 20 -- if modification is used. The present invention contemplates all types of toxin modification, including chemical and heat treatment of the toxin. In one embodiment, glutaraldehyde treatment of the toxin is contemplated. In an alternative embodiment, formaldehyde treatment of the toxin is contemplated.

25 It is not intended that the present invention be limited to a particular mode of immunization: the present invention contemplates all modes of immunization, including subcutaneous, intramuscular, intraperitoneal, and intravenous or intravascular injection, as well as *per os* administration of immunogen.

The present invention further contemplates immunization with or without adjuvant. As 30 used herein, the term "adjuvant" is defined as a substance known to increase the immune response to other antigens when administered with other antigens. If adjuvant is used, it is not intended that the present invention be limited to any particular type of adjuvant -- or that the same adjuvant, once used, be used all the time. While the present invention contemplates all types of adjuvant, whether used separately or in combinations, the preferred use of

adjuvant is the use of Complete Freund's Adjuvant followed sometime later with Incomplete Freund's Adjuvant. The invention also contemplates the use of fowl adjuvant commercially available from RIBI, as well as Quil A adjuvant commercially available from Accurate Chemical and Scientific Corporation, and Gerbu adjuvant also commercially available (GmDP: C.C. Biotech Corp.).

When immunization is used, the present invention contemplates a wide variety of immunization schedules. In one embodiment, a chicken is administered toxin(s) on day zero and subsequently receives toxin(s) in intervals thereafter. It is not intended that the present invention be limited by the particular intervals or doses. Similarly, it is not intended that the present invention be limited to any particular schedule for collecting antibody. The preferred collection time is sometime after day 35.

Where birds are used and collection of antibody is performed by collecting eggs, the eggs may be stored prior to processing for antibody. It is preferred that eggs be stored at 4°C for less than one year.

It is contemplated that chicken antibody produced in this manner can be buffer-extracted and used analytically. While unpurified, this preparation can serve as a reference for activity of the antibody prior to further manipulations (e.g., immunoaffinity purification).

III. Increasing The Effectiveness Of Antibodies

When purification is used, the present invention contemplates purifying to increase the effectiveness of both non-mammalian antitoxins and mammalian antitoxins. Specifically, the present invention contemplates increasing the percent of toxin-reactive immunoglobulin. The preferred purification approach for avian antibody is polyethylene glycol (PEG) separation.

The present invention contemplates that avian antibody be initially purified using simple, inexpensive procedures. In one embodiment, chicken antibody from eggs is purified by extraction and precipitation with PEG. PEG purification exploits the differential solubility of lipids (which are abundant in egg yolks) and yolk proteins in high concentrations of PEG 8000 (Polson *et al.*, Immunol. Comm. 9:495 [1980]). The technique is rapid, simple, and relatively inexpensive and yields an immunoglobulin fraction that is significantly more pure, in terms of contaminating non-immunoglobulin proteins than the comparable ammonium sulfate fractions of mammalian sera and horse antibodies. The majority of the PEG is removed from the precipitated chicken immunoglobulin by treatment with ethanol. Indeed,

PEG-purified antibody is sufficiently pure that the present invention contemplates the use of PEG-purified antitoxins in the passive immunization of intoxicated humans and animals.

IV. Treatment

5 The present invention contemplates antitoxin therapy for humans and other animals intoxicated by bacterial toxins. A preferred method of treatment is by parenteral administration of antitoxin.

A. Dosage Of Antitoxin

10 It was noted by way of background that a balance must be struck when administering currently available antitoxin which is usually produced in large animals such as horses: sufficient antitoxin must be administered to neutralize the toxin, but not so much antitoxin as to increase the risk of untoward side effects. These side effects are caused by: i) patient sensitivity to foreign (e.g., horse) proteins; ii) anaphylactic or immunogenic properties of non-15 immunoglobulin proteins; iii) the complement fixing properties of mammalian antibodies; and/or iv) the overall burden of foreign protein administered. It is extremely difficult to strike this balance when, as noted above, the degree of intoxication (and hence the level of antitoxin therapy needed) can only be approximated.

20 The present invention contemplates significantly reducing side effects so that this balance is more easily achieved. Treatment according to the present invention contemplates reducing side effects by using PEG-purified antitoxin from birds.

25 In one embodiment, the treatment of the present invention contemplates the use of PEG-purified antitoxin from birds. The use of yolk-derived, PEG-purified antibody as antitoxin allows for the administration of: 1) non (mammalian)-complement-fixing, avian antibody; 2) a less heterogeneous mixture of non-immunoglobulin proteins; and 3) less total protein to deliver the equivalent weight of active antibody present in currently available antitoxins. The non-mammalian source of the antitoxin makes it useful for treating patients who are sensitive to horse or other mammalian sera.

30 As is true in cases of botulism, the degree of an individual's exposure to *E. coli* toxin and the prognosis are often difficult to assess, and depend upon a number of factors (e.g., the quantity of contaminated food ingested, the toxigenicity and serotype of *E. coli* strain ingested, etc.). Thus, the clinical presentation of a patient is usually a more important consideration than a quantitative diagnostic test, for determination of dosage in antitoxin

administration. Indeed, for many toxin-associated diseases (*e.g.*, botulism, tetanus, diphtheria, etc.), there is no rapid, quantitative test to detect the presence of the toxin or organism. Rather, these toxin-associated diseases are medical emergencies which mandate immediate treatment. Confirmation of the etiologic agent must not delay the institution of therapy, as the condition of an affected patient may rapidly deteriorate. In addition to the initial treatment with antitoxin, subsequent doses may be indicated, as the patient's disease progresses. The dosage and timing of these subsequent doses is dependent upon the signs and symptoms of disease in each individual patient.

It is contemplated that the administration of antitoxin to an affected individual would involve an initial injection of an approximately 10 ml dose of immune globulin (with less than approximately 1 gram of total protein). In one preferred embodiment, it is contemplated that at least 50% of the initial injection comprises immune globulin. It is also contemplated that more purified immune globulin be used for treatment, wherein approximately 90% of the initial injection comprises immune globulin. When more purified immune globulin is used, it is contemplated that the total protein will be less than approximately 100 milligrams. It is also contemplated that additional doses be given, depending upon the signs and symptoms associated with *E. coli* verotoxin disease progression.

B. Delivery Of Antitoxin

Although it is not intended to limit the route of delivery, the present invention contemplates a method for antitoxin treatment of bacterial intoxication in which delivery of antitoxin is parenteral or oral.

In one embodiment, antitoxin is parenterally administered to a subject in an aqueous solution. It is not intended that the parenteral administration be limited to a particular route. Indeed, it is contemplated that all routes of parenteral administration will be used. In one embodiment, parenteral administration is accomplished via intramuscular injection. In an alternative embodiment, parenteral administration is accomplished via intravenous injection.

In another embodiment, antitoxin is delivered in a solid form (*e.g.*, tablets). In an alternative embodiment antitoxin is delivered in an aqueous solution. When an aqueous solution is used, the solution has sufficient ionic strength to solubilize antibody protein, yet is made palatable for oral administration. The delivery solution may also be buffered (*e.g.*, carbonate buffer, pH 9.5) which can neutralize stomach acids and stabilize the antibodies when the antibodies are administered orally. In one embodiment the delivery solution is an

aqueous solution. In another embodiment the delivery solution is a nutritional formula. Preferably, the delivery solution is infant or a dietary supplement formula (e.g., Similac®, Ensure®, and Enfamil®). Yet another embodiment contemplates the delivery of lyophilized antibody encapsulated or microencapsulated inside acid-resistant compounds.

5 Methods of applying enteric coatings to pharmaceutical compounds are well known to the art (companies specializing in the coating of pharmaceutical compounds are available: for example, The Coating Place [Verona, WI] and AAI [Wilmington, NC]). Enteric coatings which are resistant to gastric fluid and whose release (*i.e.*, dissolution of the coating to release the pharmaceutical compound) is pH dependent are commercially available (for example, the 10 polymethacrylates Eudragit® L and Eudragit® S [Röhm Tech Inc., Malden, MA]). Eudragit® S is soluble in intestinal fluid from pH 7.0; this coating can be used to microencapsulate lyophilized antitoxin antibodies and the particles are suspended in a solution having a pH above or below pH 7.0 for oral administration. The microparticles will remain intact and undissolved until they reached the intestines where the intestinal pH would cause 15 them to dissolve thereby releasing the antitoxin.

The invention contemplates a method of treatment which can be administered for treatment of acute intoxication. In one embodiment, antitoxin is administered orally in either a delivery solution or in tablet form, in therapeutic dosage, to a subject intoxicated by the bacterial toxin which served as immunogen for the antitoxin. In another embodiment of treatment of acute intoxication, a therapeutic dosage of the antitoxin in a delivery solution, is 20 parenterally administered.

The invention also contemplates a method of treatment which can be administered prophylactically. In one embodiment, antitoxin is administered orally, in a delivery solution, in therapeutic dosage, to a subject, to prevent intoxication of the subject by the bacterial toxin which served as immunogen for the production of antitoxin. In another embodiment, 25 antitoxin is administered orally in solid form such as tablets or as microencapsulated particles. Microencapsulation of lyophilized antibody using compounds such as Eudragit® (Rohm GmbH) or polyethylene glycol, which dissolve at a wide range of pH units, allows the oral administration of solid antitoxin in a liquid form (*i.e.*, a suspension) to recipients unable to tolerate administration of tablets (e.g., children or patients on feeding tubes). In one preferred embodiment the subject is a child. In another embodiment, antibody raised against whole 30 bacterial organism is administered orally to a subject, in a delivery solution, in therapeutic

dosage. In yet another preferred embodiment of prophylactic treatment, a therapeutic dosage of the antitoxin in a delivery solution, is parenterally administered.

V. Multivalent Vaccines Against *E. coli* Strains

The invention contemplates the generation of multivalent vaccines for the protection of an organism (particularly humans) against several *E. coli* strains. Of particular interest is a vaccine which stimulates the production of a humoral immune response to *E. coli* O157:H7, O26:H11, O113:H21, O91:H21, and O111:NM, in humans. The antigens comprising the vaccine preparation may be native or recombinantly produced toxin proteins from the *E. coli* serotypes listed above. When native toxin proteins are used as immunogens they are generally modified to reduce the toxicity. It is contemplated that glutaraldehyde-modified toxin proteins will be used. In an alternative embodiment, formaldehyde-modified toxin proteins will be used.

The invention contemplates that recombinant *E. coli* verotoxin proteins be used in conjunction with either native toxins or toxoids from other organisms as antigens in a multivalent vaccine preparation. It is also contemplated that recombinant *E. coli* toxin proteins be used in the multivalent vaccine preparation.

VI. Detection Of Toxin

The invention contemplates detecting bacterial toxin in a sample. The term "sample" in the present specification and claims is used in its broadest sense. On the one hand it is meant to include a specimen or culture (e.g., microbiological cultures). On the other hand, it is meant to include both biological and environmental samples.

Biological samples may be animal, including human, fluid, solid (e.g., stool) or tissue, as well as liquid and solid food and feed products and ingredients such as dairy items, vegetables, meat and meat by-products, and waste. Biological samples may be obtained from all of the various families of common domestic animals, including but not limited to bovines (e.g. cattle), ovines (e.g., sheep), caprines (e.g., goats), porcines (e.g., swine), equines (e.g., horses), canines (e.g., dogs), lagamorphs (e.g., rabbits), and felines (e.g., cats), etc. It is also intended that samples may be obtained from feral or wild animals, including, but not limited to, such animals as ungulates (e.g., deer), bear, fish, lagamorphs, rodents, etc.

Environmental samples include environmental material such as surface matter, soil, water and industrial samples, as well as samples obtained from food and dairy processing

instruments, apparatus, equipment, utensils, disposable and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention.

The invention contemplates detecting bacterial toxin by a competitive immunoassay method that utilizes recombinant toxin VT1 and toxin VT2 proteins, antibodies raised against recombinant bacterial toxin proteins. A fixed amount of the recombinant toxin proteins are immobilized to a solid support (e.g., a microtiter plate) followed by the addition of a biological sample suspected of containing a bacterial toxin. The biological sample is first mixed with affinity-purified or PEG fractionated antibodies directed against the recombinant toxin protein. A reporter reagent is then added which is capable of detecting the presence of antibody bound to the immobilized toxin protein. The reporter substance may comprise an antibody with binding specificity for the antitoxin attached to a molecule which is used to identify the presence of the reporter substance. If toxin is present in the sample, this toxin will compete with the immobilized recombinant toxin protein for binding to the anti-recombinant antibody thereby reducing the signal obtained following the addition of the reporter reagent. A control is employed where the antibody is not mixed with the sample. This gives the highest (or reference) signal.

The invention also contemplates detecting bacterial toxin by a "sandwich" immunoassay method that utilizes antibodies directed against recombinant bacterial toxin proteins. Affinity-purified antibodies directed against recombinant bacterial toxin proteins are immobilized to a solid support (e.g., microtiter plates). Biological samples suspected of containing bacterial toxins are then added followed by a washing step to remove substantially all unbound antitoxin. The biological sample is next exposed to the reporter substance, which binds to antitoxin and is then washed free of substantially all unbound reporter substance. The reporter substance may comprise an antibody with binding specificity for the antitoxin attached to a molecule which is used to identify the presence of the reporter substance. Identification of the reporter substance in the biological tissue indicates the presence of the bacterial toxin.

It is also contemplated that bacterial toxin be detected by pouring liquids (e.g., soups and other fluid foods and feeds including nutritional supplements for humans and other animals) over immobilized antibody which is directed against the bacterial toxin. It is contemplated that the immobilized antibody will be present in or on such supports as cartridges, columns, beads, or any other solid support medium. In one embodiment, following

the exposure of the liquid to the immobilized antibody, unbound toxin is substantially removed by washing. The liquid is then exposed to a reporter substance which detects the presence of bound toxin. In a preferred embodiment the reporter substance is an enzyme, fluorescent dye, or radioactive compound attached to an antibody which is directed against the 5 toxin (*i.e.*, in a "sandwich" immunoassay). It is also contemplated that the detection system will be developed as necessary (*e.g.*, the addition of enzyme substrate in enzyme systems; observation using fluorescent light for fluorescent dye systems; and quantitation of radioactivity for radioactive systems).

10 EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the disclosure which follows, the following abbreviations apply: °C (degrees Centigrade); rpm (revolutions per minute); BSA (bovine serum albumin); ELISA (enzyme-linked immunosorbent assay); IgG (immunoglobulin G); IgY (immunoglobulin Y); IP (intraperitoneal); SC (subcutaneous); H₂O (water); HCl (hydrochloric acid); LD₁₀₀ (lethal dose for 100% of experimental animals); aa (amino acid); HPLC (high performance liquid chromatography); kDa (kilodaltons); gm (grams); µg (micrograms); mg (milligrams); ng (nanograms); µl (microliters); ml (milliliters); mm (millimeters); nm (nanometers); µm (micrometer); M (molar); mM (millimolar); MW (molecular weight); sec (seconds); min(s) (minute/minutes); hr(s) (hour/hours); MgCl₂ (magnesium chloride); NaCl (sodium chloride); Na₂CO₃ (sodium carbonate); OD₂₈₀ (optical density at 280 nm); OD₆₀₀ (optical density at 600 nm); PAGE (polyacrylamide gel electrophoresis); PBS [phosphate buffered saline (150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2)]; PEG (polyethylene glycol); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl)aminomethane); w/v (weight to volume); v/v (volume to volume); Amicon (Amicon, Inc., Beverly, MA); Amresco (Amresco, Inc., Solon, OH); ATCC (American Type Culture Collection, Rockville, MD); BBL (Baltimore Biologics Laboratory, (a division of Becton Dickinson), Cockeysville, MD); Becton Dickinson (Becton Dickinson Labware, Lincoln Park, NJ); BioRad (BioRad, Richmond, CA); Biotech (C-C 20 Biotech Corp., Poway, CA); Charles River (Charles River Laboratories, Wilmington, MA); Falcon (*e.g.* Baxter Healthcare Corp., McGaw Park, IL and Becton Dickinson); Fisher Biotech (Fisher Biotech, Springfield, NJ); GIBCO (Grand Island Biologic Company/BRL, Grand 25 Island, NY); Mallinckrodt (a division of Baxter Healthcare Corp., McGaw Park, IL);

Millipore (Millipore Corp., Marlborough, MA); New England Biolabs (New England Biolabs. Inc.. Beverly. MA); Novagen (Novagen, Inc., Madison. WI); Pharmacia (Pharmacia, Inc.. Piscataway, NJ); Qiagen (Qiagen, Chatsworth, CA); Showdex (Showa Denko America. Inc.. New York, NY); Sigma (Sigma Chemical Co., St. Louis. MO); RIBI (RIBI Immunochemical Research Inc.. Hamilton. MT); Accurate Chemical and Scientific Corp. (Accurate Chemical and Scientific Corp.. Hicksville, NY); Kodak (Eastman-Kodak. Rochester. NY); and Stratagene (Stratagene. La Jolla. CA).

When a recombinant protein is described in the specification it is referred to in a short-hand manner by the amino acids in the toxin sequence present in the recombinant protein rounded to the nearest 10. The specification gives detailed construction details for all recombinant proteins such that one skilled in the art will know precisely which amino acids are present in a given recombinant protein.

The first set of Examples (Examples 1-5) was designed to develop an antidote to *E. coli* O157:H7 verotoxins and evaluate its effectiveness *in vitro* and *in vivo*. In the first experiments, high titer verotoxin antibodies were generated in laying hens hyperimmunized with chemically detoxified and/or native verotoxins. These Laying hens were immunized with either recombinant *E. coli* O157:H7 VT1 or VT2 (rVT1 and rVT2) treated with glutaraldehyde and mixed with adjuvant.

Next, toxin-reactive polyclonal antibodies were isolated by bulk fractionation from egg yolks pooled from hyperimmunized hens. Large quantities of polyclonal antibodies (IgY) were harvested from resulting eggs using a two-step polyethylene glycol fractionation procedure.

Third, the immunoreactivity and yields of VT IgY were analyzed by analytical immunochemical methods (e.g., enzyme immunoassay (EIA) and Western blotting). EIA and Western blot analysis showed that the resulting egg preparations contained high titer IgY that reacted with both the immunizing and the heterologous toxins (i.e., rVT1 IgY reacted against both rVT1 and rVT2, and vice versa).

Fourth, VT neutralization potency was analyzed *in vitro* using a Vero cytotoxicity assay. Vero cytotoxicity of rVT1 and rVT2 could be completely inhibited by VT IgY. These antibodies also demonstrated substantial verotoxin cross-neutralization.

Fifth, the efficacy of passively administered avian verotoxin antibodies in preventing the lethal effects of verotoxin poisoning was assessed in a mouse disease model. Toxin neutralizing antibodies were administered by parenteral dosing regimens to assess the most

effective strategy for therapeutic intervention. Efficacy of verotoxin antibodies was demonstrated using multiple murine disease models. In these experiments, antibodies prevented both the morbidity and lethality of homologous and heterologous toxins using a toxin/antitoxin premix format; mice infected orally with a lethal dose of viable *E. coli* O157:H7 were protected from both morbidity and lethality when treated parenterally four hours post-infection with either rVT1 or rVT2 antibodies; and mice given a lethal dose of *E. coli* O91:H21 (a particularly virulent strain which only produces VT2c, a VT2 structural variant) and treated parenterally *up to 10 hours later* with rVT1 IgY administered parenterally were protected from both morbidity and lethality.

10

EXAMPLE 1

TOXIN ANALYSIS AND IMMUNIZATION

Purified recombinant *E. coli* O157:H7 verotoxins, rVT1 and rVT2, were obtained from Denka Sieken Co., Ltd. (Tokyo, Japan). Toxin genes were isolated, inserted into expression plasmids, and expressed in *E. coli*. Recombinant proteins were then purified by ammonium sulfate precipitation, ion exchange chromatography on DEAE Sephadex and hydroxyapatite, and gel filtration chromatography by the supplier. Upon receipt, toxins were analyzed to verify identity, purity and toxicity, as described below.

20 A. **Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).**

Samples of each toxin (2 µg) were heat-denatured in a buffer containing SDS and β -mercaptoethanol followed by electrophoresis on 10–20% gradient gels (Bio-Rad, Richmond, CA). Resolved polypeptide bands were visualized using the silver stain procedure of C.R. Merril, *et al.*, "Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins," *Science* 211: 1437-1438 (1981).

25 VT1 and VT2 are each composed of subunit A and multiple copies of subunit B. Subunit A is often nicked into fragments A1 and A2 which are linked by a disulfide bridge. As shown in Figure 1, when separated by SDS-PAGE in the presence of β -mercaptoethanol, rVT1 resolved into 3 bands that corresponded to subunit A (~31 Kda), fragment A1 (~27 Kda) and a mixture of subunit B and fragment A2 (~4 Kda). Similarly, rVT2 resolved into subunit A (~33 Kda), fragment A1 (~27 Kda) and a mixture of subunit B and fragment A2 (~8 Kda) (Figure 1). In this Figure, rVT1 is in Lane 1, and rVT2 is in Lane 2: the positions of

molecular weight markers (Kda) are shown at the left. VT component polypeptides are identified at the right.

These results are consistent with previous reports of VT1 and VT2 purified from naturally occurring toxigenic strains (V. V. Padhye *et al.*, "Purification and Physicochemical Properties of a Unique Vero Cell Cytotoxin From *Escherichia coli* O157:H7." *Biochem. Biophys. Res. Commun.* 139: 424-430 [1986]; and F. B. Kittel *et al.*, "Characterization and inactivation of verotoxin 1 produced by *Escherichia coli* O157:H7." *J. Agr. Food Chem.* 39: 141-145 [1991]).

10 **B. High Performance Liquid Chromatography (HPLC).**

Chromatography was performed at room temperature (RT) under isocratic conditions using a Waters 510 HPLC pump. Eluted protein was measured using a Waters 490E programmable multi-wavelength detector (Millipore Corp., Milford, MA). The VT's were separated on an 8 x 300 mm (ID) Shodex KW803 column, using 10 mM sodium phosphate, 0.15 M NaCl, pH 7.4 (phosphate buffered saline [PBS]) as the mobile phase at a flow rate of 1 ml/min.

15 The purity of non-denatured rVT's was assessed by HPLC. As shown in the chromatographs in Figure 2, each toxin eluted at approximately 10 min. as a single absorbance peak at 280 nm. By integration of the area under each peak, the rVT's were shown to be >99% pure.

20 **C. Vero Cell Cytotoxicity Assay.**

Cytotoxic activity of rVT1 and rVT2 was assessed using modified procedures of Padhye, *et al.* (V. V. Padhye *et al.*, "Purification and Physicochemical Properties of a Unique Vero Cell Cytotoxin From *Escherichia coli* O157:H7." *Biochem. Biophys. Res. Commun.* 139: 424-430 [1986]), and McGee, *et al.*, (Z. A. McGee, *et al.*, "Local induction of tumor necrosis factor as molecular mechanism of mucosal damage by gonococci." *Microbial Pathogenesis* 12: 333-341 [1992]). Microtiter plates (96 well, Falcon, Microtest III) were inoculated with approximately 1×10^4 Vero cells (ATCC, CCL81) per well (100 μ l) and incubated overnight at 37°C in the presence of 5% CO₂ to form Vero cell monolayers. rVT1 and rVT2 solutions were serially diluted in Medium 199 supplemented with 5% fetal bovine serum (Life Technologies, Grand Island, NY), added to each well of the microtiter plates and incubated at 37°C for 18-24 hrs. Adherent (viable) cells were stained with 0.2% crystal

violet (Mallinckrodt) in 2% ethanol. Excess stain was rinsed away and the stained cells were solubilized by adding 100 μ l of 1% SDS to each well. Absorbance of each well was measured at 570 nm. and the percent cytotoxicity of each test sample was calculated using the following formula:

5

$$\% \text{ Vero Cytotoxicity} = [1 - (\text{Absorbance Sample}/\text{Absorbance Control})] \times 100$$

To determine whether the rVT's possessed potency equivalent to published cytotoxicity values, a Vero cell cytotoxicity assay was performed (Figure 3). Between 0.01–10,000 pg of either rVT1 or rVT2 was added to Vero cells. The amounts of rVT causing 50% cell death (CD_{50}), as calculated by second degree polynomial curve fitting were 0.97 pg and 1.5 pg. for rVT1 and rVT2, respectively. These results are consistent with CD_{50} values reported previously for naturally occurring VT1 and VT2 in the range 1–35 pg and 1–25 pg, respectively (M. Petric *et al.*, "Purification and biological properties of *Escherichia coli* verocytotoxin." FEMS Microbiol. Lett., 41: 63-68 [1987]; V. L. Tesh, *et al.*, "Comparison of relative toxicities of Shiga-Like toxins Type I and Type II for mice." Infect. Immun., 61: 3392-3402 [1993]; N. Dickie *et al.*, "Purification of an *Escherichia coli* Serogroup O157:H7 verotoxin and its detection in North American hemorrhagic colitis isolates." J. Clin. Microbiol., 27: 1973-1978 [1989]; and U. Kongmuang, *et al.*, "A simple method for 15 purification of Shiga or Shiga-Like toxin from *Shigella dysenteriae* and *Escherichia coli* O157:H7 by immunoaffinity chromatography." FEMS Microbiol. Lett., 48: 379-383 [1987]). It has been observed that toxicity is lost with storage, explaining why higher amounts of toxin 20 were used in the neutralization assays described below.

25 **D. Mouse Lethal Dose Determination.**

To verify rVT1 and rVT2 toxicity, male (20–22 g) CD-1 mice were injected intraperitoneally with varying amounts of rVT1 or rVT2 in 200 μ L phosphate buffer. Doses were selected based on published LD_{50} values for VT1 and VT2 in CD-1 mice. To minimize the sacrifice of live animals, a full statistical toxin LD_{50} was not determined. Mice were 30 observed for morbidity and mortality over 7-day period.

Further confirmation of rVT toxicity was obtained from mouse lethality experiments (Table 2). Mice were injected intraperitoneally with varying amounts of either rVT1 or rVT2 and observed 7 days for mortality. Within 72–120 hrs. post-injection, all of the mice died

from 100 ng of rVT1 or 10 ng of rVT2, respectively. This lethality study served as a verification of expected toxicity but not as a statistical determination of LD₅₀. Nonetheless, these results were consistent with toxicity studies which reported LD₅₀ values in CD-1 mice of 0.4–2.0 µg for purified VT1 and 0.001–1.0 µg for purified VT2 (V. L. Tesh, *et al.*,

5 "Comparison of relative toxicities of Shiga-Like toxins Type I and Type II for mice." Infect. Immun. 61: 3392-3402 [1993]; and A. D. O'Brien. and G. D. LaVeck. "Purification and characterization of *Shigella dysenteriae* 1-like toxin produced by *Escherichia coli*," Infect. Immun. 40: 675-683 [1983]).

10

Table 2.
Lethality of rVT1 in CD-1 Mice

15

20

ng VT1 Injected	Survivors/Total	Hours Post-Injection
100	7/7	24 ± 2
	5/7	48 ± 2
	0/7	72 ± 2
10	7/7	24 ± 2
	7/7	48 ± 2
	7/7	72 ± 2
1.0	6/6	24 ± 2
	6/6	48 ± 2
	6/6	72 ± 2

Table 3.
Lethality of rVT2 in CD-1 Mice

ng VT2 Injected	Survivors/Total	Hours Post-Injection
5	3/6	48 ± 2
	2/6	72 ± 2
	0/6	120 ± 2
10	5/6	48 ± 2
	4/6	72 ± 2
	0/6	120 ± 2
10	6/6	48 ± 2
	6/6	72 ± 2
	6/6	120 ± 2

15 The recombinant toxins used in these studies thus appeared to contain protein components and toxicities consistent with literature reports for native toxins. Based on these structural and functional analyses, the rVT's were considered suitable as antigens to generate specific avian antibodies.

20 **E. Antigen Preparation.**

25 Lyophilized samples, rVT1 and rVT2 were received and each was reconstituted with 2.5 mL of deionized water to a final concentration of 100 µg/ml in phosphate buffer. To form a toxoid, the solutions were then treated with 0.4% glutaraldehyde (Mallinckrodt) at 4°C overnight and stored at -20°C thereafter. When needed, toxoid was thawed and mixed 5:1 (volume:volume) with GERBU adjuvant (C. C. Biotech Corporation, Poway, CA). White Leghorn laying hens were injected subcutaneously with 25 µg of either rVT1 or rVT2 toxoid in adjuvant at 2-3 week intervals.

EXAMPLE 2

30 **PEG EXTRACTION OF EGG YOLK ANTIBODY**

Hyperimmune eggs were collected after 3 immunizations with toxoid. Egg yolks were separated from whites, pooled according to their immunogen group and blended with 4 volumes of 10 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS). Polyethylene glycol

8000 (PEG) (Amresco, Solon, OH) was then added to a final concentration of 3.5% and the mixture centrifuged at 10,000 x g for 10 min. to remove the precipitated lipid fraction. IgY-rich supernatant was filtered through cheesecloth and PEG was again added to a final concentration of 12%. The solution was centrifuged as above and the resulting supernatant discarded. The IgY pellet was then dissolved in PBS to either the original (1X PEG IgY) or 1/4 of the original (4X PEG IgY) yolk volume, filtered through a 0.45 μ membrane and stored at 4°C.

EXAMPLE 3

ANTITOXIN IMMUNOASSAYS

A. Enzyme Immunoassay (EIA).

EIA was used to monitor antibody responses during the immunization course. Wells of 96-well Pro-Bind microtiter plates (Falcon, through Scientific Products, McGaw Park, IL) were each coated with 1 μ g of rVT's (not toxoid) in PBS overnight at 2-8°C. Wells were washed 3 times with PBS containing 0.05% Tween-20 (PBS-T) to remove unbound antigen, and the remaining protein binding sites were blocked with PBS containing 1 mg/ml BSA for 60 min. at room temperature (RT). IgY, diluted in PBS, was then added to the wells and incubated for 1 hr. at 37°C. Wells were washed as before to remove unbound primary antibody and incubated for 1 hr. at 37°C with alkaline phosphatase-conjugated rabbit-anti-chicken IgG (Sigma Chemical Company, St. Louis, MO) diluted 1:1000 in PBS-T. Wells were again washed and 1 mg/ml *p*-nitrophenyl phosphate (Sigma Chemical Company, St. Louis, MO) in 50 mM Na₂CO₃, 10 mM MgCl₂, pH 9.5 was added and allowed to incubate at RT. Phosphatase activity was detected by absorbance at 410 nm using a Dynatech MR700 microtiter plate reader.

Laying Leghorn hens were immunized as described above (Example 1, part E), using glutaraldehyde-treated rVT's. Following several immunizations, eggs were collected and IgY harvested by PEG fractionation. Figures 4 and 5 show rVT1 or rVT2 specific antibody responses detected using EIA at dilutions of the original yolk IgY concentration of 1:30,000 and 1:6,000, respectively. IgY fractionated similarly from unimmunized hens (*i.e.*, preimmune antibody) did not react with either antigen at test dilutions above 1:50. Although these EIA results indicate significant antibody responses, prior experience with other toxin antigens has shown that optimization of immunization regimens, including increasing the amount of

antigen, can yield titers in excess of 1:100,000 (B. S. Thalley, *et al.*, "Development of an Avian Antitoxin to Type A Botulinum Neurotoxin," in Botulinum and Tetanus Neurotoxins: Neurotransmission and Biomedical Aspects, B. R. DasGupta, (ed.) [Plenum Press, New York, 1993] pp. 467-472). As may be expected due to their structural homology and consistent with previous reports (e.g., V. V. Padhye *et al.*, "Production and characterization of monoclonal antibodies to verotoxins 1 and 2 from *Escherichia coli* O157:H7," *J. Agr. Food Chem.*, 39: 141-145 [1989]; S. C. Head *et al.*, "Purification and characterization of verocytotoxin 2," *FEMS Microbiol. Lett.*, 51: 211-216 [1988]; and N. C. Strockbine *et al.*, "Characterization of Monoclonal Antibodies against Shiga-Like Toxin from *Escherichia coli*," *Infect. Immun.*, 50: 695-700 [1985]). Figures 4 and 5 also demonstrate that antibodies generated against one toxin cross-reacted *in vitro* with the other toxin.

B. Western Blot Analysis.

Western blots (Figure 6) performed to determine the reactivity of rVT antibodies against constituent VT polypeptides showed that rVT1 and rVT2 antibodies reacted with subunit A and fragment A1 of either toxin, and with subunit B and fragment A2 of rVT1 only. In this Figure, Panel A contains preimmune IgY, Panel B contains rVT1 IgY, and Panel C contains rVT2 IgY. Lane 1 in each panel contains rVT1 (2 μ g) and Lane 2 contains rVT2 (2 μ g). Preimmune IgY was largely nonreactive to either rVT. Both rVT IgY preparations, however, failed to react with subunit B and fragment A2 of rVT2. Some explanations for this lack of measurable reactivity might include poor immunogenicity, denaturation of the immunogen during glutaraldehyde treatment, loss of conformational epitopes due to detergent or reducing agent, or poor transfer to nitrocellulose.

To resolve the high and low molecular weight components, 2 μ g each of rVT1 and rVT2 were separated by SDS-PAGE (described above) and then transferred to nitrocellulose paper using the Milliblot-SDE system (Millipore, Medford, MA) according to the manufacturer's instructions. Paper strips were stained temporarily with Ponceau S (Sigma Chemical Company, St. Louis, MO) to visualize the polypeptides and then blocked overnight in PBS containing 5% dry milk. Each strip was agitated gently in IgY diluted in PBS-T for 2 hrs. at RT. Strips were each washed with three changes of PBS-T to remove unbound primary antibody and incubated for 2 hrs. at RT with goat anti-chicken alkaline phosphatase (Kirkegaard and Perry, Gaithersburg, MD) diluted 1:500 in PBS-T containing 1 mg/ml BSA. The blots were washed as before and rinsed in 50 mM Na₂CO₃, pH 9.5. Strips were

submerged in alkaline-phosphatase substrate (5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (Kirkegaard and Perry) until sufficient signal was observed. Color development was stopped by flooding the blots with water.

5

EXAMPLE 4

IN VITRO TOXIN NEUTRALIZATION: VERO CELL ASSAY

IgY neutralization of rVT1 and rVT2 was assessed using the modified Vero cytotoxicity assay described above (Example 1, part C). Various concentrations of IgY, 10 diluted in Medium 199 supplemented with 5% fetal bovine serum (GIBCO), were mixed with sufficient toxin to cause 50% cell death and allowed to incubate at 37°C for 60 minutes. These toxin/antibody mixtures were then added to Vero cell-coated microtiter plate wells according to the procedure described above (Example 1, part C).

15 The toxin neutralization capacity of the rVT antibodies was analyzed first using a Vero cell toxicity assay. The results in Figure 7 show that rVT1 IgY neutralized completely the cytotoxic activity of rVT1 at an endpoint dilution of 1/320. Furthermore, rVT2 IgY neutralized the heterologous rVT1 toxin, but at a higher endpoint concentration.

In a similar experiment (see Figure 8), rVT1 and rVT2 antibodies were each able to 20 neutralize rVT2 at equivalent endpoint dilutions. This strong cross-neutralization correlates with the observed strong cross-reactivity of VT1 IgY with VT2 A seen on Western blots (Figure 6). These results show that IgY antibodies are able to neutralize effectively VT cytotoxicity and that the antibodies can cross-neutralize structurally-related heterologous toxins.

25

EXAMPLE 5

TOXIN NEUTRALIZATION: MOUSE ASSAYS

A. Toxin Challenge Model.

IgY in PBS was premixed with a lethal dose of toxin (as determined above) and 30 injected intraperitoneally into male CD-1 (20-22 gm) mice. Mice were observed for a 7-day period for signs of intoxication such as ruffled fur, huddling and disinclination to move, followed by hind leg paralysis, rapid breathing and death. Untreated, infected mice usually died within 12 hrs. after signs of severe illness (i.e., within 48-72 hrs. post-injection).

Once it was demonstrated that rVT antibodies were able to neutralize rVT cytotoxicity *in vitro*, protection experiments were next performed in mice. First, animals were challenged with rVT premixed with rVT IgY to determine whether toxin lethality could be neutralized under conditions optimal for antigen/antibody reaction. Tables 4 and 5 show that antibodies premixed with the homologous toxin (*e.g.*, rVT1 with rVT1 IgY) prevented lethality of rVT. Preimmune IgY was unable to neutralize either toxin in these studies.

Table 4
Neutralization of rVT1 Using rVT IgY

100 ng rVT2 Premixed*	Survivors/Total	<i>p</i>
Preimmune Antibody	0/12	
rVT1 Antibody	12/12	< 0.001
rVT2 Antibody	12/12	< 0.001

15 *Toxin was pre-mixed with IgY and incubated for 1 hour at room temperature prior to administration.

Table 5
Neutralization of rVT2 Using rVT IgY

10 ng rVT1 Premixed*	Survivors/Total	<i>p</i>
Preimmune Antibody	0/12	
rVT1 Antibody	12/12	< 0.001
rVT2 Antibody	12/12	< 0.001

25 *Toxin was pre-mixed with IgY and incubated for 1 hour at room temperature prior to administration.

Antibodies premixed with the heterologous toxin (*e.g.*, rVT2 with rVT1 IgY) also prevented lethality *in vivo*. These data are in contrast to previous observations where rabbit 30 polyclonal antibodies generated against either toxin were cross-reactive with the heterologous toxin by EIA and Western blot, but were unable to neutralize the heterologous toxin in either Vero cell cytotoxicity and mouse lethality assays (S. C. Head, *et al.*, "Serological differences between verocytotoxin 2 and Shiga-like toxin II," Lancet ii: 751 [1988]; S. C. Head *et al.*, "Purification and characterization of verocytotoxin 2," FEMS Microbiol. Lett., 51: 211-216

[1988]; N. C. Strockbine *et al.*, "Characterization of Monoclonal Antibodies against Shiga-Like Toxin from *Escherichia coli*," Infect. Immun., 50: 695-700 [1985]; and V. V. Padhye *et al.*, "Purification and Physicochemical Properties of a Unique Vero Cell Cytotoxin From *Escherichia coli* O157:H7," Biochem. Biophys. Res. Commun., 139: 424-430 [1986]).

5 However, Head *et al.* showed that VT2 B-subunit specific monoclonal antibodies neutralized VT1 weakly in a Vero cytotoxicity assay (S. C. Head, *et al.*, "Serological differences between verocytotoxin 2 and Shiga-like toxin II," Lancet ii: 751 [1988]). In a report by Donohue-Rolfe, *et al.*, a VT2 B subunit-specific monoclonal antibody neutralized both VT1 and VT2 completely in a Hela cytotoxicity assay (A. Donohue-Rolfe *et al.*, 10 "Purification of Shiga toxin and Shiga-like toxins I and II by receptor analog affinity chromatography with immobilized P1 glycoprotein and production of cross reactive monoclonal antibodies," Infect. Immun., 57: 3888-3893 [1989]).

15 These results showed for the first time complete cross-neutralization in Vero cell cytotoxicity and mouse lethality assays, revealing that VT1 and VT2 do indeed share common neutralizing epitopes. These results may indicate that hens generate different antibody specificities as compared to mammals, and/or that differences in immunization methods might have maintained the immunogenicity of conformational epitopes necessary for cross-neutralization. Nonetheless, this cross-neutralization suggests that IgY antibodies may contain the range of reactivities essential for an effective antitoxin.

20

B. Viable organism infection model.

Streptomycin-resistant *E. coli* O157:H7 (strain 933 cu-rev) or *E. coli* O91:H21 (strain B2F1) (both kindly provided by Dr. Alison O'Brien, Dept. of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD) were used in a murine infection model described by Wadolkowski, *et al.* (E. A. Wadolkowski *et al.*, "Mouse model for colonization and disease caused by enterohemorrhagic *Escherichia coli* O157:H7," Infect. Immun., 58: 2438-2445 [1990]). Organisms were grown in Luria broth and incubated overnight at 37°C in an Environ Shaker (Lab Line, Melrose Park, IL) (T. Maniatis *et al.*, 25 Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y., [1982]). Bacterial suspensions were centrifuged at 6700 x g for 5 minutes. The resulting pellet was then washed twice with sterile PBS and resuspended in sterile 20% (w/v) sucrose. Five to 8 week-old male CD-1 mice were provided drinking water containing 30 5 mg/ml streptomycin sulfate *ad libitum* for 24 hrs. Food and water were then withheld for

another 16-18 hrs. after which mice were challenged orally with 10^{10} streptomycin-resistant *E. coli* O157:H7 or O91:H21. Mice were housed individually and permitted food and water containing 5 mg/ml streptomycin sulfate. IgY was injected intraperitoneally at varying times post-infection and animals observed for both morbidity and mortality for 10 days.

5 To monitor bacterial colonization in animals, 1 gram of feces was collected, homogenized, and plated onto MacConkey agar medium (Difco Laboratories, Detroit, MI) containing 100 μ g/ml streptomycin and incubated at 37°C as described by Wadolkowski, *et al.* (E. A. Wadolkowski *et al.*, "Mouse model for colonization and disease caused by enterohemorrhagic *Escherichia coli* O157:H7," *Infect. Immun.* 58: 2438-2445 [1990]). The 10 serotype of *E. coli* O157:H7, 933 cu-rev excreted in feces was confirmed by slide agglutination with O- and H-specific antisera (Difco Laboratories, Detroit, MI).

15 Kidneys were removed from experimental animals and fixed in 10% buffered neutral formalin. Sections of parafilm-embedded tissue were stained with hematoxylin and eosin (General Medical Laboratories, Madison, WI) and examined by light microscopy. All tissue sections were coded to avoid bias before microscopic examination to determine renal pathology.

The toxin neutralization ability of rVT IgY was further studied using a streptomycin-treated CD-1 mouse infection model. This model was chosen because it produces definitive systemic pathology and reproducible mortality.

20 In contrast to previous studies by Wadolkowski, *et al.* (E. A. Wadolkowski *et al.*, "Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shiga-like toxin Type II," *Infect. Immun.* 58: 3959-3965 [1990]), where mice were given subunit-specific monoclonal antibodies *prior* to infection, the mice in this study were inoculated orally with 2×10^{10} viable *E. coli* O157:H7 (strain 933 cu-rev) and treated 25 with rVT IgY 4 hrs. *following* inoculation. Fecal cultures showed that 10^7 - 10^8 challenge organisms per gram of feces were shed throughout the course of the experiment, thus confirming that infection was established. Tables 6 and 7 show that animals treated with either rVT1 or rVT2 IgY were protected from lethality caused by infection ($p<0.01$ and $p<0.001$, respectively) and that preimmune IgY failed to provide protection to the mice.

30

Table 6
Protection of Mice From *E. coli* O157:H7
With rVT1 IgY

IgY Treatment	Survivors/Total	p	Morbidity/Total
Preimmune Antibody	0/5		5/5
rVT1 Antibody	9/10	< 0.01	1/10

*IgY was administered intraperitoneally 4 hours following infection, and once daily for 10 days thereafter.

Table 7
Protection of Mice From *E. coli* O157:H7
With rVT2 IgY

IgY Treatment	Survivors/Total	p	Morbidity/Total
Preimmune Antibody	0/6		6/6
rVT2 Antibody	10/10	< 0.005	0/10

*IgY was administered intraperitoneally 4 hours following infection, and once daily for 10 days thereafter.

Renal histopathology (see Figure 9) of the control (preimmune IgY) animals showed dilation, degeneration and renal tubular necrosis with no glomerular damage. This is consistent with previous reports showing that renal tubular involvement occurs predominantly in this streptomycin-treated mouse infectivity model (E. A. Wadolkowski *et al.*, "Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shiga-like toxin Type II," *Infect. Immun.*, 58: 3959-3965 [1990]). Importantly, none of the survivors exhibited similar signs of morbidity though treated with IgY 4 hrs. *after* infection (see Figure 9).

Furthermore, avian antibodies generated against rVT1 were able to prevent both mortality and morbidity in a mouse model where VT2 alone is implicated in the pathogenesis and lethality of *E. coli* O157:H7 strain 933 cu-rev (E. A. Wadolkowski *et al.*, "Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shiga-like toxin Type II," *Infect. Immun.*, 58: 3959-3965 [1990]).

To assess the broader utility of the IgY verotoxin antibodies in treating VTEC-associated disease, the mouse infectivity study was performed using a more virulent VTEC serotype known to produce VT2c—a structural variant of VT2—but not VT1 (S. W. Lindgren

et al., "Virulence of enterohemorrhagic *Escherichia coli* O91:H21 clinical isolates in an orally infected mouse model." *Infect. Immun.* 61: 3832-3842 [1993]).

Mice were inoculated orally with 5×10^9 *E. coli* O91:H21 (strain B2F1) and treated subsequently with IgY. Notably, the heterologous rVT1 IgY protected strongly against the 5 lethal effects of the VT2c structural variant, even when administered as long as 10 hrs. following infection (Table 8). Ten hours was the longest treatment window tested in this study. Only 1 of the 8 animals treated with rVT1 IgY died ($p < 0.02$), and those that survived showed no overt signs of renal histopathology (*i.e.*, acute bilateral tubular necrosis). It can thus be concluded that rVT1 IgY completely neutralized toxicity of VT2c, indicating its 10 potential as a therapeutic for at least one other pathogenic VTEC.

15 **Table 8**
Protection of Mice From *E. coli* O91:H21
With rVT1 IgY

IgY Treatment	Survivors/Total	<i>p</i>	Morbidity/Total
Preimmune Antibody	0/7		7/7
rVT1 Antibody	7/8	< 0.02	1/8

20 *IgY was administered intraperitoneally 10 hours following infection, and once daily for 8 days thereafter.

25 These Examples highlight several important findings supporting the feasibility of using verotoxin antitoxin. First, polyclonal IgY generated against either VT1 or VT2 from *E. coli* O157:H7, cross-reacted with and fully cross-neutralized the toxicity of the non-immunizing toxin both *in vitro* and *in vivo*. Second, recombinant toxins fully neutralized the toxicity of naturally-occurring toxins produced by *E. coli* O157:H7 during the course of infection. Third, 30 antibodies generated against rVT1 from *E. coli* O157:H7 could prevent morbidity and mortality in mice infected orally with lethal doses of *E. coli* O91:H21, a particularly virulent strain which only produces VT2c, suggesting their utility in preventing systemic sequelae. Because VT1 is identical to Shiga-toxin (A. D. O'Brien *et al.*, "Shiga and Shiga-like toxins. Microbial Rev., 51: 206-220 [1987]), VT antibodies may also be useful in preventing complications stemming from *Shigella dysenteriae* infection. Finally, animals treated with VT

IgY were protected against both death and kidney damage when treated as long as 10 hrs. after infection, supporting the hypothesis that a window for antitoxin intervention exists.

These studies strongly support the use of parenterally-administered, toxin-specific IgY as a antitoxin to prevent life-threatening complications associated with *E. coli* O157:H7 and other VTEC infections. It is contemplated that this approach would be most useful in preventing HUS and other complications when administered after the onset of bloody diarrhea and before the presentation of systemic disease.

The VT IgY developed in these studies were shown to react with and neutralize both recombinant and naturally-occurring VT. The antibody titers as measured by EIA are indicative of reasonable antibody production in the hen, however much higher production levels can be obtained with larger immunizing doses.

The results from these Examples clearly demonstrate the feasibility and provide the experimental basis for development of an avian antidote for *E. coli* O157:H7 verotoxins suitable for use in humans. In contrast to previous reports showing that rabbit polyclonal VT1 and VT2 antibodies cross-reacted, but did not cross-neutralize the heterologous toxin in Vero cytotoxicity or in mouse lethality studies (e.g., V. V. Padhye *et al.*, "Production and characterization of monoclonal antibodies to verotoxins 1 and 2 from *Escherichia coli* O157:H7," *J. Agr. Food Chem.*, 39: 141-145 [1989]; S. C. Head *et al.*, "Purification and characterization of verocytotoxin 2," *FEMS Microbiol. Lett.*, 51: 211-216 [1988]; and N. C. Strockbine *et al.*, "Characterization of monoclonal antibodies against Shiga-like toxin from *Escherichia coli*," *Infect. Immun.*, 50: 695-700 [1985]), these data provide the first demonstration of cross-neutralization *in vivo*. Antibodies against one toxin neutralized completely the heterologous toxin in both Vero cytotoxicity and mouse lethality assays. Both rVT1 and rVT2 antibodies also prevented morbidity (as assessed by renal histopathology) and mortality in mice infected with lethal doses of *E. coli* O157:H7 – the etiologic agent in 90% of the documented cases of hemolytic uremic syndrome (HUS) in the U.S. (P. M. Griffin and R. V. Tauxe, "The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome," *Epidemiol. Rev.*, 13: 60 [1990]). With at least two other VTEC serotypes known to cause HUS, the finding that rVT1 antibodies neutralized a VT2 variant produced by *E. coli* O91:H21 suggests that avian polyclonal antibodies may provide an effective antidote against other verotoxin-producing *E. coli*. These data also show for the first time, that antibodies may be administered *after* infection and still protect against morbidity and mortality.

EXAMPLE 6

EXPRESSION OF TOXIN GENES

The previous Examples clearly showed that avian polyclonal antibodies to recombinant toxins protected animals infected with verotoxigenic *E. coli*. This Example includes 5 expression of toxin genes (A and B subunits alone and together as whole toxins) in suitable prokaryotic expression systems to achieve high levels of VT antigen production.

The sequence of the toxin gene has been determined (see e.g., M.P. Jackson *et al.*, "Nucleotide sequence analysis and comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from *Escherichia coli* 933," 44:109 10 [1987]). The coding regions of the A and B subunits of VT-1 are listed in SEQ ID NOS:1 and 3, respectively. The corresponding amino acid sequence of the A and B subunits of the VT-1 toxin are listed in SEQ ID NOS:2 and 4, respectively. The coding regions of the A and B subunits of VT-2 are listed in SEQ ID NOS:5 and 7, respectively. The corresponding 15 amino acid sequence of the A and B subunits of the VT-2 toxin are listed in SEQ ID NOS:6 and 8, respectively. In addition, SEQ ID NOS:9 and 10 list the sequences which direct the expression of a poly-cistronic RNA capable of directing the translation of both the A and B subunits from the VT-1 and VT-2 genes, respectively.

In choosing a strategy for recombinant VT antigen production, there are three primary 20 technical factors to consider. First, the appropriate VT antigen components representing the spectrum of toxin epitopes encountered in nature must be utilized. Second, the protein antigens must be expressed at sufficient levels and purity to enable immunization and large-scale antibody purification. Third, the neutralizing epitopes must be preserved in the immunogen and immunoabsorbant. Approaches that offer the greatest promise for high level expression of periplasmically localized, native, affinity-tagged proteins were developed.

25 Figure 10 shows the fusion constructs of VT components and affinity tags.

A. Expression of affinity-tagged C-terminal constructs.

The VT1 and VT2 A and B subunits (SEQ ID NOS:1, 3, 5 and 7) are cloned into the 30 pET-23b vector (Novagen). This vector is designed to allow expression of native proteins containing C-terminal poly-His tags. The vector utilizes a strong T7 polymerase promoter to drive high level expression of target proteins. The methionine initiation codon is engineered to contain a unique *Nde*I restriction enzyme site (CATATG). The VT1 and VT2 genes are engineered to convert the signal sequence methionine codon into a *Nde*I site utilizing PCR

5 mutagenesis. PCR primers were designed which contain the sequence GCCAT fused to the first 20-24 bases of the genes (starting at the ATG start codon of the signal tag; SEQ ID NOS:12-19, see Table below). Upon PCR amplification, the 5' start codon of each gene is converted to an *Nde*I site, compatible with the pET-23 vector-encoded *Nde*I site, allowing cloning of the amplified genes into the vector without the addition of vector-encoded amino acids.

10 Primers containing the C-terminal 7 codons of each gene (21 bases) fused to the sequence CTCGAGCC were synthesized, in order to add a C-terminal poly-His tag to each gene. The underlined bases are an *Xba*I site, that is compatible with the *Xba*I site of the pET-23 vector. These primers precisely delete the native stop codons, and when cloned into the pET-23 vector, add a C-terminal extension of "LeuGluHisHisHisHisHis" (SEQ ID NO: 11). The following table lists the primer pairs are utilized to create PCR fragments containing the A and B subunits derived from VT-1 and VT-2 toxin genes suitable for insertion into the pET-23b vector.

15

Table 9**Primers**

Toxin Gene and Subunit	N-terminal Primer	C-terminal Primer
VT-1 Subunit A	SEQ ID NO:12	SEQ ID NO:13
VT-1 Subunit B	SEQ ID NO:14	SEQ ID NO:15
20 VT-2 Subunit A	SEQ ID NO:16	SEQ ID NO:17
VT-2 Subunit B	SEQ ID NO:18	SEQ ID NO:19
VT-1 Subunits A and B	SEQ ID NO:12	SEQ ID NO:15
VT-2 Subunits A and B	SEQ ID NO:16	SEQ ID NO:19

25

Thus, utilizing PCR amplification with the above modified N- and C-terminal primers, the A and B subunits of VT1 and VT2 are expressed as proteins containing an 8 amino acid C-terminal extension bearing an poly-histidine affinity tag. The amino acid sequence of the histidine-tagged VT-1 A subunit produced by expression from the pET-23b vector is listed in SEQ ID NO:21 (the associated DNA sequence is listed in SEQ ID NO:20); the amino acid sequence of the histidine-tagged VT-1 B subunit is listed in SEQ ID NO:23 (the associated

30

DNA sequence is listed in SEQ ID NO:22); the amino acid sequence of the histidine-tagged VT-2 A subunit is listed in SEQ ID NO:25 (the associated DNA sequence is listed in SEQ ID NO:24); the amino acid sequence of the histidine-tagged VT-2 B subunit is listed in SEQ ID NO:27 (the associated DNA sequence is listed in SEQ ID NO:26).

5 Both subunits may be expressed from a single expression constructs by utilizing SEQ ID NOS:12 and 15 to prime synthesis of the VT-1 toxin gene and SEQ ID NOS:16 and 19 to prime synthesis of the VT-2 toxin gene. The resulting PCR products are cleaved with *Nde*I and *Xho*I, as described for the cloning of the subunit genes into the pET-23b vector. Expression of the A and B subunits from such an expression vector, results in the expression 10 of a native A subunit and a his-tagged B subunit. As the A and B subunits assemble into a complex, the presence of the his-tag on only the B subunit is sufficient to allow purification of the holotoxin on metal chelate columns as described below.

15 The proofreading *Pfu* polymerase (Stratagene) is utilized for PCR amplification to reduce the error rate during amplification. Genomic DNA from an *E. coli* O157:H7 strain is utilized as template DNA. Following the PCR, the amplification products are digested with *Nde*I and *Xho*I and cloned into the pCR-Script SK cloning vehicle (Stratagene) to permit 20 DNA sequence analysis of the amplified products. The DNA sequence analysis is performed to ensure that no base changes are introduced during amplification. Once the desired clones are identified by DNA sequencing, the inserts are then excised utilizing *Nde*I and *Xho*I, and cloned into a similarly cut pET-23b vector to create the expression constructs. According to the published sequences, neither the VT1 nor VT2 genes contain either of these restriction sites.

25 The poly-His-tagged proteins produced by expression of the VT-1 and VT-2 gene sequences in the pET-23b constructs are then purified by IMAC. This method uses metal-chelate affinity chromatography to purify native or denatured proteins which have histidine tails (see e.g., K. J. Petty, "Metal-Chelate Affinity Chromatography," in Current Protocols in Molecular Biology, Supplement 24, Unit 10.11B [1993]).

B. Expression of Toxin Containing N-terminal Affinity Tags

30 Two expression systems, pMal-p2 and pFLAG-1 are utilized to attach an N-terminal affinity tag to the A subunits from the VT-1 and VT-2 toxins.

MBP-tagged constructs. To construct A chains containing the maltose binding protein (MBP) at the N-terminus of the A subunit, PCR amplified gene products are cloned into the

5 pMal-p2 vector (New England Biolabs) as C-terminal fusions to a periplasmically-secreted version of the MBP. The MBP selectively binds to amylose resins and serves as an affinity tag on the MBP/A subunit fusion protein. The pMal-p2 vector contains an engineered factor Xa cleavage site, which permits the removal of the affinity tag (*i.e.*, MBP) from the fusion protein after purification.

10 The MBP/A subunit fusions are generated as follows. The VT1 and VT2 A subunits are PCR-amplified utilizing the following DNA primers. SEQ ID NOS:28-31: SEQ ID NOS:28 and 29 comprise the 5' and 3' primers, respectively, for the amplification of the VT1 A subunit; SEQ ID NOS:30 and 31 comprise the 5' and 3' primers, respectively, for the 15 amplification of the VT2 A subunit. In both cases, the 5' or N-terminal primer contains the sequence CGGAATTC fused to the first codon of the mature polypeptide (rather than the start of the signal peptide, since the MBP signal peptide is utilized). These 5' primers contain an engineered *Eco*RI site that is not contained internally in either gene, that is compatible with the *Eco*RI site of the pMal-p2 vector. The 3' or C-terminal primers incorporate an *Xba*I site 20 as described above for the generation of the His-tagged toxins, but in this case, the 3' primer is designed to include the natural termination codon of the A subunits.

25 The genes are amplified, cloned into pCR-Script SK, and sequenced as described above. The inserts are then excised with *Eco*RI and *Xba*I, and cloned into *Eco*RI/*Sal*I-cleaved pMal-p2 vector (*Sal*I and *Xba*I sites are compatible). This construct allows expression and secretion of the VT1 and VT2 A subunit genes as C-terminal fusions with MBP. The amino acid sequence of the MBP/VT-1A fusion protein is listed in SEQ ID NO:33 (the associated DNA sequence is listed in SEQ ID NO:32). The amino acid sequence of the MBP/VT-2A fusion protein is listed in SEQ ID NO:35 (the associated DNA sequence is listed in SEQ ID NO:34).

30 The resulting fusion proteins are then affinity purified on an amylose column and the bound fusion protein is eluted under mild conditions by competition with maltose. The MBP N-terminal-tagged A subunits are cleaved with factor Xa and the MBP is removed by chromatography on an amylose column. The resulting A subunits which contain a 4 amino acid N-terminal extension are then used as immunogens.

Flag tag constructs. In an alternative embodiment, the VT1 and VT2 A subunit genes are engineered to contain the "flag tag" through the use of the pFLAG-1 vector system. The flag tag is located between the *OmpA* secretion signal sequence and the authentic N-

terminus of the target protein in the pFlag-1 vector. To construct N-terminal flag-tagged A chains, the *EcoRI/XbaI* A subunit PCR fragments (generated as described above for the MBP fusion proteins) are cloned into identically cleaved pFlag-1 vector (Eastman-Kodak), to produce an expression construct utilizing the *OmpA* signal peptide for secretion of A subunit fusion proteins containing the flag peptide at the N-terminus. After secretion, the periplasmic protein contains the N-terminal 8 amino acid flag tag, followed by 4 vector-encoded amino acids fused to the recombinant A subunit. The amino acid sequence of the flag tag/VT-1 A subunit fusion protein is listed in SEQ ID NO:37 (the associated DNA sequence is listed in SEQ ID NO:36). The amino acid sequence of the flag tag/VT-2 A subunit fusion protein is listed in SEQ ID NO:39 (the associated DNA sequence is listed in SEQ ID NO:38).

The flag tag fusion proteins are then purified by immunoaffinity chromatography utilizing a calcium-dependent monoclonal antibody (Antiflag M1; Eastman-Kodak). Mild elution of purified protein is achieved by chelating the calcium in the column buffer with ethylenediamine tetraacetic acid (EDTA).

15

C. Evaluation of fusion construct expression.

The fusion constructs described above are expressed in *E. coli* strain BL21, or T7 polymerase-containing derivatives [e.g., BL21(DE3), BL21(DE3) pLysS, BL21(DE3)pLysE] (Novagen) for pET plasmids, and periplasmically-secreted recombinant protein purified by affinity chromatography. Recombinant proteins are analyzed for correct conformation by testing the following parameters:

25

- a) It is believed that the B subunit must associate into pentamers to be conformationally correct. This is assessed by reducing and native SDS-PAGE analyses of native and chemically-cross-linked proteins and sizing HPLC;
- b) It is believed that a properly folded A subunit is expected to retain its native enzymatic activity. This is tested by its capacity to inhibit protein synthesis in an *in vitro* toxicity assay;
- c) It is believed that *in vitro* toxicity of assembled recombinant holotoxin is compared to commercially available holotoxins to determine whether recombinant A and B subunits can assemble into functional holotoxin. The

purified N-terminal-tagged A subunits (after cleavage and purification from MBP or untreated flag-tagged proteins) are combined *in vitro* with the corresponding B chains, and their toxicity evaluated utilizing a quantitative microtiter cytotoxicity assay, such as that described by M.K. Gentry and M. 5 Dalrymple, "Quantitative Microtiter Cytotoxicity Assay for *Shigella* Toxin." *J. Clin. Microbiol.*, 12:361-366 (1980).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: OPHIDIAN PHARMACEUTICALS, INC.
- (ii) TITLE OF INVENTION: TREATMENT FOR VEROTOXIN-PRODUCING E. COLI
- (iii) NUMBER OF SEQUENCES: 39
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: MEDLEN & CARROLL
 - (B) STREET: 220 MONTGOMERY STREET, SUITE 2200
 - (C) CITY: SAN FRANCISCO
 - (D) STATE: CALIFORNIA
 - (E) COUNTRY: UNITED STATES OF AMERICA
 - (F) ZIP: 94104
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: CARROLL, PETER G.
 - (B) REGISTRATION NUMBER: 32,837
 - (C) REFERENCE/DOCKET NUMBER: OPHD-02171
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (415) 705-8410
 - (B) TELEFAX: (415) 397-8338

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 945 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..945
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATG AAA ATA ATT ATT TTT AGA GTG CTA ACT TTT TTC TTT GTT ATC TTT Met Lys Ile Ile Ile Phe Arg Val Leu Thr Phe Phe Phe Val Ile Phe	48
1 5 10 15	
TCA GTT AAT GTG GTG GCG AAG GAA TTT ACC TTA GAC TTC TCG ACT GCA Ser Val Asn Val Val Ala Lys Glu Thr Leu Asp Phe Ser Thr Ala	96
20 25 30	
AAG ACG TAT GTA GAT TCG CTG AAT GTC ATT CGC TCT GCA ATA GGT ACT Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr	144
35 40 45	
CCA TTA CAG ACT ATT TCA TCA GGA GGT ACG TCT TTA CTG ATG ATT GAT Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp	192
50 55 60	

AGT GGC TCA GGG GAT AAT TTG TTT GCA GTT GAT GTC AGA GGG ATA GAT Ser Gly Ser Gly Asp Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp 65 70 75 80	240
GCA GAG GAA GGG CGG TTT AAT AAT CTA CGG CTT ATT GTT GAA CGA AAT Ala Glu Glu Gly Arg Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn 85 90 95	288
AAT TTA TAT GTG ACA GGA TTT GTT AAC AGG ACA AAT AAT GTT TTT TAT Asn Leu Tyr Val Thr Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr 100 105 110	336
CGC TTT GCT GAT TTT TCA CAT GTT ACC TTT CCA GGT ACA ACA GCG GTT Arg Phe Ala Asp Phe Ser His Val Thr Phe Pro Gly Thr Thr Ala Val 115 120 125	384
ACA TTG TCT GGT GAC AGT AGC TAT ACC ACG TTA CAG CGT GTT GCA GGG Thr Leu Ser Gly Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly 130 135 140	432
ATC AGT CGT ACG GGG ATG CAG ATA AAT CGC CAT TCG TTG ACT ACT TCT Ile Ser Arg Thr Gly Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser 145 150 155 160	480
TAT CTG GAT TTA ATG TCG CAT AGT GGA ACC TCA CTG ACG CAG TCT GTG Tyr Leu Asp Leu Met Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val 165 170 175	528
GCA AGA GCG ATG TTA CGG TTT GTT ACT GTG ACA GCT GAA GCT TTA GCT Ala Arg Ala Met Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg 180 185 190	576
TTT CGG CAA ATA CAG AGG GGA TTT CGT ACA ACA CTG GAT GAT CTC AGT Phe Arg Gln Ile Gln Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser 195 200 205	624
GGG CGT TCT TAT GTA ATG ACT GCT GAA GAT GTT GAT CTT ACA TTG AAC Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn 210 215 220	672
TGG GGA AGG TTG AGT AGC GTC CTG CCT GAC TAT CAT GGA CAA GAC TCT Trp Gly Arg Leu Ser Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser 225 230 235 240	720
GTT CGT GTA GGA AGA ATT TCT TTT GGA AGC ATT AAT GCA ATT CTG GGA Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly 245 250 255	768
AGC GTG GCA TTA ATA CTG AAT TGT CAT CAT GCA TCG CGA GTT GCC Ser Val Ala Leu Ile Leu Asn Cys His His His Ala Ser Arg Val Ala 260 265 270	816
AGA ATG GCA TCT GAT GAG TTT CCT TCT ATG TGT CCG GCA GAT GGA AGA Arg Met Ala Ser Asp Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg 275 280 285	864
GTC CGT GGG ATT ACG CAC AAT AAA ATA TTG TGG GAT TCA TCC ACT CTG Val Arg Gly Ile Thr His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu 290 295 300	912
GGG GCA ATT CTG ATG CGC AGA ACT ATT AGC AGT Gly Ala Ile Leu Met Arg Arg Thr Ile Ser Ser 305 310 315	945

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 315 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Lys Ile Ile Ile Phe Arg Val Leu Thr Phe Phe Phe Val Ile Phe
 1 5 10 15

Ser Val Asn Val Val Ala Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala
 20 25 30

Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr
 35 40 45

Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp
 50 55 60

Ser Gly Ser Gly Asp Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp
 65 70 75 80

Ala Glu Glu Gly Arg Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn
 85 90 95

Asn Leu Tyr Val Thr Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr
 100 105 110

Arg Phe Ala Asp Phe Ser His Val Thr Phe Pro Gly Thr Thr Ala Val
 115 120 125

Thr Leu Ser Gly Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly
 130 135 140

Ile Ser Arg Thr Gly Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser
 145 150 155 160

Tyr Leu Asp Leu Met Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val
 165 170 175

Ala Arg Ala Met Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg
 180 185 190

Phe Arg Gln Ile Gln Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser
 195 200 205

Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn
 210 215 220

Trp Gly Arg Leu Ser Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser
 225 230 235 240

Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly
 245 250 255

Ser Val Ala Leu Ile Leu Asn Cys His His His Ala Ser Arg Val Ala
 260 265 270

Arg Met Ala Ser Asp Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg
 275 280 285

Val Arg Gly Ile Thr His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu
 290 295 300

Gly Ala Ile Leu Met Arg Arg Thr Ile Ser Ser
 305 310 315

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 267 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..267

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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AGT GCG CTG GCG ACG CCT GAT TGT GTA ACT GGA AAG GTG GAG TAT ACA	96
Ser Ala Leu Ala Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr	
20 25 30	
AAA TAT AAT GAT GAC GAT ACC TTT ACA GTT AAA GTG GGT GAT AAA GAA	144
Lys Tyr Asn Asp Asp Asp Thr Phe Thr Val Lys Val Gly Asp Lys Glu	
35 40 45	
TTA TTT ACC AAC AGA TGG AAT CTT CAG TCT CTT CTT CTC AGT GCG CAA	192
Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu Ser Ala Gln	
50 55 60	
ATT ACG GGG ATG ACT GTA ACC ATT AAA ACT AAT GCC TGT CAT AAT GGA	240
Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly	
65 70 75 80	
GGG GGA TTC AGC GAA GTT ATT TTT CGT	267
Gly Gly Phe Ser Glu Val Ile Phe Arg	
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 89 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Lys Lys Thr Leu Leu Ile Ala Ala Ser Leu Ser Phe Phe Ser Ala	
1 5 10 15	
Ser Ala Leu Ala Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr	
20 25 30	
Lys Tyr Asn Asp Asp Asp Thr Phe Thr Val Lys Val Gly Asp Lys Glu	
35 40 45	
Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu Ser Ala Gln	
50 55 60	
Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly	
65 70 75 80	
Gly Gly Phe Ser Glu Val Ile Phe Arg	
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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 954 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 1..954

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATG AAG TGT ATA TTA TTT AAA TGG GTA CTG TGC CTG TTA CTG GGT TTT Met Lys Cys Ile Leu Phe Lys Trp Val Leu Cys Leu Leu Gly Phe 1 5 10 15	48
TCT TCG GTA TCC TAT TCC CGG GAG TTT ACG ATA GAC TTT TCG ACC CAA Ser Ser Val Ser Tyr Ser Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln 20 25 30	96
CAA AGT TAT GTC TCT TCG TTA AAT AGT ATA CGG ACA GAG ATA TCG ACC Gln Ser Tyr Val Ser Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr 35 40 45	144
CCT CTT GAA CAT ATA TCT CAG GGG ACC ACA TCG GTG TCT GTT ATT AAC Pro Leu Glu His Ile Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn 50 55 60	192
CAC ACC CAC GGC AGT TAT TTT GCT GTG GAT ATA CGA GGG CTT GAT GTC His Thr His Gly Ser Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val 65 70 75 80	240
TAT CAG GCG CGT TTT GAC CAT CTT CGT CTG ATT ATT GAG CAA AAT AAT Tyr Gln Ala Arg Phe Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn 85 90 95	288
TTA TAT GTG GCA GGG TTC GTT AAT ACG GCA ACA AAT ACT TTC TAC CGT Leu Tyr Val Ala Gly Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg 100 105 110	336
TTT TCA GAT TTT ACA CAT ATA TCA GTG CCC GGT GTG ACA ACG GTT TCC Phe Ser Asp Phe Thr His Ile Ser Val Pro Gly Val Thr Thr Val Ser 115 120 125	384
ATG ACA ACG GAC AGC AGT TAT ACC ACT CTG CAA CGT GTC GCA GCG CTG Met Thr Thr Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu 130 135 140	432
GAA CGT TCC GGA ATG CAA ATC AGT CGT CAC TCA CTG GTT TCA TCA TAT Glu Arg Ser Gly Met Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr 145 150 155 160	480
CTG GCG TTA ATG GAG TTC AGT GGT AAT ACA ATG ACC AGA GAT GCA TCC Leu Ala Leu Met Glu Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser 165 170 175	528
AGA GCA GTT CTG CGT TTT GTC ACT GTC ACA GCA GAA GCC TTA CGC TTC Arg Ala Val Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe 180 185 190	576
AGG CAG ATA CAG AGA GAA TTT CGT CAG GCA CTG TCT GAA ACT GCT CCT Arg Gln Ile Gln Arg Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro 195 200 205	624
GTG TAT ACG ATG ACG CCG GGA GAC GTG GAC CTC ACT CTG AAC TGG GGG Val Tyr Thr Met Thr Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly 210 215 220	672

CGA ATC AGC AAT GTG CTT CCG GAG TAT CGG GGA GAG GAT GGT GTC AGA Arg Ile Ser Asn Val Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg 225 230 235 240	720
GTG GGG AGA ATA TCC TTT AAT AAT ATA TCA GCG ATA CTG GGG ACT GTG Val Gly Arg Ile Ser Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val 245 250 255	768
GCC GTT ATA CTG AAT TGC CAT CAT CAG GGG GCG CGT TCT GTT CGC GCC Ala Val Ile Leu Asn Cys His His Gln Gly Ala Arg Ser Val Arg Ala 260 265 270	816
GTG AAT GAA GAG AGT CAA CCA GAA TGT CAG ATA ACT GGC GAC AGG CCT Val Asn Glu Glu Ser Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro 275 280 285	864
GTT ATA AAA ATA AAC AAT ACA TTA TGG GAA AGT AAT ACA GCT GCA GCG Val Ile Lys Ile Asn Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala 290 295 300	912
TTT CTG AAC AGA AAG TCA CAG TTT TTA TAT ACA ACG GGT AAA Phe Leu Asn Arg Lys Ser Gln Phe Leu Tyr Thr Gly Lys 305 310 315	954

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 318 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Lys Cys Ile Leu Phe Lys Trp Val Leu Cys Leu Leu Leu Gly Phe 1 5 10 15
Ser Ser Val Ser Tyr Ser Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln 20 25 30
Gln Ser Tyr Val Ser Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr 35 40 45
Pro Leu Glu His Ile Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn 50 55 60
His Thr His Gly Ser Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val 65 70 75 80
Tyr Gln Ala Arg Phe Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn 85 90 95
Leu Tyr Val Ala Gly Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg 100 105 110
Phe Ser Asp Phe Thr His Ile Ser Val Pro Gly Val Thr Thr Val Ser 115 120 125
Met Thr Thr Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu 130 135 140
Glu Arg Ser Gly Met Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr 145 150 155 160
Leu Ala Leu Met Glu Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser 165 170 175
Arg Ala Val Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe 180 185 190

Arg Gln Ile Gln Arg Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro
 195 200 205
 Val Tyr Thr Met Thr Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly
 210 215 220
 Arg Ile Ser Asn Val Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg
 225 230 235 240
 Val Gly Arg Ile Ser Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val
 245 250 255
 Ala Val Ile Leu Asn Cys His His Gln Gly Ala Arg Ser Val Arg Ala
 260 265 270
 Val Asn Glu Glu Ser Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro
 275 280 285
 Val Ile Lys Ile Asn Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala
 290 295 300
 Phe Leu Asn Arg Lys Ser Gln Phe Leu Tyr Thr Gly Lys
 305 310 315

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 267 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..267
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATG AAG AAG ATG TTT ATG GCG GTT TTA TTT GCA TTA GCT TCT GTT AAT	48
Met Lys Lys Met Phe Met Ala Val Leu Phe Ala Leu Ala Ser Val Asn	
1 5 10 15	
GCA ATG GCG GCG GAT TGT GCT AAA GGT AAA ATT GAG TTT TCC AAG TAT	96
Ala Met Ala Ala Asp Cys Ala Lys Gly Lys Ile Glu Phe Ser Lys Tyr	
20 25 30	
AAT GAG GAT GAC ACA TTT ACA GTG AAG GTT GAC GGG AAA GAA TAC TGG	144
Asn Glu Asp Asp Thr Phe Thr Val Lys Val Asp Gly Lys Glu Tyr Trp	
35 40 45	
ACC AGT CGC TGG AAT CTG CAA CCG TTA CTG CAA AGT GCT CAG TTG ACA	192
Thr Ser Arg Trp Asn Leu Gln Pro Leu Leu Gln Ser Ala Gln Leu Thr	
50 55 60	
GGA ATG ACT GTC ACA ATC AAA TCC AGT ACC TGT GAA TCA GGC TCC GGA	240
Gly Met Thr Val Thr Ile Lys Ser Ser Thr Cys Glu Ser Gly Ser Gly	
65 70 75 80	
TTT GCT GAA GTG CAG TTT AAT AAT GAC	267
Phe Ala Glu Val Gln Phe Asn Asn Asp	
85	

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 89 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Lys Lys Met Phe Met Ala Val Leu Phe Ala Leu Ala Ser Val Asn
 1 5 10 15

Ala Met Ala Ala Asp Cys Ala Lys Gly Lys Ile Glu Phe Ser Lys Tyr
 20 25 30

Asn Glu Asp Asp Thr Phe Thr Val Lys Val Asp Gly Lys Glu Tyr Trp
 35 40 45

Thr Ser Arg Trp Asn Leu Gln Pro Leu Leu Gln Ser Ala Gln Leu Thr
 50 55 60

Gly Met Thr Val Thr Ile Lys Ser Ser Thr Cys Glu Ser Gly Ser Gly
 65 70 75 80

Phe Ala Glu Val Gln Phe Asn Asn Asp
 85

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1241 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATGAAAATAA TTATTTTAG AGTGCTAACT TTTTTCTTG TTATCTTTC AGTTAATGTG	60
GTGGCGAAGG AATTTACCTT AGACTTCTCG ACTGCAAAGA CGTATGTAGA TTGCTGAAT	120
GTCATTGCT CTGCAATAGG TACTCCATTA CAGACTATT CATCAGGAGG TACGTCTTA	180
CTGATGATTG ATAGTGGCTC AGGGATAAT TTGTTGCAG TTGATGTCAG AGGGATAGAT	240
GCAGAGGAAG GGCGGTTAA TAATCTACGG CTTATTGTT AACGAAATAA TTTATATGTG	300
ACAGGATTG TTAACAGGAC AAATAATGTT TTTTATCGCT TTGCTGATT TTCACATGTT	360
ACCTTCCAG GTACAACAGC GGTTACATTG TCTGGTGACA GTAGCTATAC CACGTTACAG	420
CGTGGTGCAG GGATCAGTCG TACGGGGATG CAGATAAAC CGCATTGTT GACTACTTCT	480
TATCTGGATT TAATGTCGCA TAGTGGAACC TCACTGACGC AGTCTGTGGC AAGAGCGATG	540
TTACGGTTG TTACTGTGAC AGCTGAAGCT TTACGTTTC GGCAAATACA GAGGGGATT	600
CGTACAACAC TGGATGATCT CAGTGGCGT TCTTATGTAA TGACTGCTGA AGATGTTGAT	660
CTTACATTGA ACTGGGGAAG GTTGAGTAGC GTCCTGCCTG ACTATCATGG ACAAGACTCT	720
GTTCGTGTAG GAAGAATTTC TTTTGGAAAGC ATTAATGCAA TTCTGGGAAG CGTGGCATT	780
ATACTGAATT GTCATCATCA TGCATCGCGA GTTGCCAGAA TGGCATCTGA TGAGTTCCCT	840
TCTATGTGTC CGGCAGATGG AAGAGTCCGT GGGATTACCGC ACAATAAAAT ATTGTGGGAT	900

TCATCCACTC TGGGGCAAT TCTGATGCGC AGAACTATTA GCAGTTAAC AGGGGGTAAA	960
TAAAGGAGTT AAGCATGAAA AAAACATTAT TAATAGCTGC ATCGCTTCA TTTTTTCAG	1020
CAAGTGCCT GGCACGCCT GATTGTGAA CTGGAAAGGT GGAGTATACA AAATATAATG	1080
ATGACGATAC CTTTACAGTT AAAGTGGGTG ATAAAGAATT ATTTACCAAC AGATGGAATC	1140
TTCAGTCTCT TCTTCTCAGT GCGAAATTA CGGGGATGAC TGTAACCATT AAAACTAATG	1200
CCTGTCATAA TGGAGGGGA TTCAGCGAAG TTATTTTCG T	1241

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1235 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATGAAGTGT AATTATTTAA ATGGGTACTG TGCCTGTTAC TGGGTTTTTC TTCGGTATCC	60
TATTCCCGGG AGTTTACGAT AGACTTTCG ACCCAACAAA GTTATGTCTC TTCGTTAAAT	120
AGTATACGGA CAGAGATATC GACCCCTCTT GAACATATAT CTCAGGGAC CACATCGGTG	180
TCTGTTATTA ACCACACCCA CGGCAGTTAT TTTGCTGTGG ATATACGAGG GCTTGATGTC	240
TATCAGGCAGC GTTTTGACCA TCTTCGTCTG ATTATTGAGC AAAATAATT ATATGTGGCA	300
GGGTTCGTTA ATACGGCAAC AAATACTTTC TACCGTTTT CAGATTTAC ACATATATCA	360
GTGCCCGGTG TGACAACGGT TTCCATGACA ACGGACAGCA GTTATACCAC TCTGCAACGT	420
GTCGAGCGC TGGAACGTT CCGGAATGCAA ATCAGTCGTC ACTCACTGGT TTCATCATAT	480
CTGGCGTTAA TGGAGTTCAAG TGGTAATACA ATGACCAGAG ATGCATCCAG AGCAGTTCTG	540
CGTTTGTCAGCTGACAGC AGAACCTTA CGCTTCAGGC AGATACAGAG AGAATTCGT	600
CAGGCAGTGT CTGAAACTGC TCCTGTGTAT ACGATGACGC CGGGAGACGT GGACCTCACT	660
CTGAACTGGG GGCGAATCAG CAATGTGCTT CCGGAGTATC GGGGAGAGGA TGGTGTCA	720
GTGGGGAGAA TATCCTTTAA TAATATATCA GCGATACTGG GGACTGTGGC CGTTATACTG	780
AATTGCCATC ATCAGGGGGC GCGTTCTGTT CGCGCCGTGA ATGAAGAGAG TCAACCAGAA	840
TGTCAGATAA CTGGCGACAG GCCTGTTATA AAAATAAACAA ATACATTATG GGAAAGTAAT	900
ACAGCTGCAG CGTTTCTGAA CAGAAAGTCA CAGTTTTAT ATACAACGGG TAAATAAGG	960
AGTTAACGAT GAAGAACATG TTTATGGCGG TTTTATTGCA ATTAGCTTCT GTTAATGCAA	1020
TGGCGCGGA TTGTGCTAAA GGTAAAATTG AGTTTTCAA GTATAATGAG GATGACACAT	1080
TTACAGTGAA GGTTGACGGG AAAGAACACT GGACCAGTCG CTGGAATCTG CAACCGTTAC	1140
TGCAAAGTGC TCAGTTGACA GGAATGACTG TCACAATCAA ATCCAGTACC TGTGAATCAG	1200
GCTCCGGATT TGCTGAAGTG CAGTTAATA ATGAC	1235

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids

- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Leu Glu His His His His His His
1 5

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCCATATGAA AATAATTATT TTTAGAGTG

29

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGCTCGAGAC TGCTAATAGT TCTGCGCAT

29

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCCATATGAA AAAAACATTA TTAATAGC

28

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGCTCGAGAC GAAAAATAAC TTCGCTGAA

29

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GCCATATGAA GTGTATATTA TTTAAATGG

29

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GGCTCGAGTT TACCCGTTGT ATATAAAAAC

30

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CGCATATGAA GAAGATGTTT ATGGCG

26

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGCTCGAGGT CATTATTAAC CTGCACTTC

29

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 969 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..969

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATG AAA ATA ATT ATT TTT AGA GTG CTA ACT TTT TTC TTT GTT ATC TTT

48

Met Lys Ile Ile Ile Phe Arg Val Leu Thr Phe Phe Phe Val Ile Phe		
1 5 10 15		
TCA GTT AAT GTG GTG GCG AAG GAA TTT ACC TTA GAC TTC TCG ACT GCA		96
Ser Val Asn Val Val Ala Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala		
20 25 30		
AAG ACG TAT GTA GAT TCG CTG AAT GTC ATT CGC TCT GCA ATA GGT ACT		144
Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr		
35 40 45		
CCA TTA CAG ACT ATT TCA TCA GGA GGT ACG TCT TTA CTG ATG ATT GAT		192
Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp		
50 55 60		
AGT GGC TCA GGG GAT AAT TTG TTT GCA GTT GAT GTC AGA GGG ATA GAT		240
Ser Gly Ser Gly Asp Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp		
65 70 75 80		
GCA GAG GAA GGG CGG TTT AAT AAT CTA CGG CTT ATT GTT GAA CGA AAT		288
Ala Glu Glu Gly Arg Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn		
85 90 95		
AAT TTA TAT GTG ACA GGA TTT GTT AAC AGG ACA AAT AAT GTT TTT TAT		336
Asn Leu Tyr Val Thr Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr		
100 105 110		
CGC TTT GCT GAT TTT TCA CAT GTT ACC TTT CCA GGT ACA ACA CGG GTT		384
Arg Phe Ala Asp Phe Ser His Val Thr Phe Pro Gly Thr Thr Ala Val		
115 120 125		
ACA TTG TCT GGT GAC AGT AGC TAT ACC ACG TTA CAG CGT GTT GCA GGG		432
Thr Leu Ser Gly Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly		
130 135 140		
ATC AGT CGT ACG GGG ATG CAG ATA AAT CGC CAT TCG TTG ACT ACT TCT		480
Ile Ser Arg Thr Gly Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser		
145 150 155 160		
TAT CTG GAT TTA ATG TCG CAT AGT GGA ACC TCA CTG ACG CAG TCT GTG		528
Tyr Leu Asp Leu Met Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val		
165 170 175		
GCA AGA GCG ATG TTA CGG TTT GTT ACT GTG ACA GCT GAA GCT TTA CGT		576
Ala Arg Ala Met Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg		
180 185 190		
TTT CGG CAA ATA CAG AGG GGA TTT CGT ACA ACA CTG GAT GAT CTC AGT		624
Phe Arg Gln Ile Gln Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser		
195 200 205		
GGG CGT TCT TAT GTA ATG ACT GCT GAA GAT GTT GAT CTT ACA TTG AAC		672
Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn		
210 215 220		
TGG GGA AGG TTG AGT AGC GTC CTG CCT GAC TAT CAT GGA CAA GAC TCT		720
Trp Gly Arg Leu Ser Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser		
225 230 235 240		
GTT CGT GTA GGA AGA ATT TCT TTT GGA AGC ATT AAT GCA ATT CTG GGA		768
Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly		
245 250 255		
AGC GTG GCA TTA ATA CTG AAT TGT CAT CAT GCA TCG CGA GTT GCC		816
Ser Val Ala Leu Ile Leu Asn Cys His His His Ala Ser Arg Val Ala		
260 265 270		
AGA ATG GCA TCT GAT GAG TTT CCT TCT ATG TGT CCG GCA GAT GGA AGA		864
Arg Met Ala Ser Asp Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg		
275 280 285		

GTC CGT GGG ATT ACG CAC AAT AAA ATA TTG TGG GAT TCA TCC ACT CTG Val Arg Gly Ile Thr His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu 290 295 300	912
GGG GCA ATT CTG ATG CGC AGA ACT ATT AGC AGT CTC GAG CAC CAC CAC Gly Ala Ile Leu Met Arg Arg Thr Ile Ser Ser Leu Glu His His His 305 310 315 320	960
CAC CAC CAC His His His	969

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 323 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Met Lys Ile Ile Ile Phe Arg Val Leu Thr Phe Phe Phe Val Ile Phe 1 5 10 15
Ser Val Asn Val Val Ala Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala 20 25 30
Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr 35 40 45
Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp 50 55 60
Ser Gly Ser Gly Asp Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp 65 70 75 80
Ala Glu Glu Gly Arg Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn 85 90 95
Asn Leu Tyr Val Thr Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr 100 105 110
Arg Phe Ala Asp Phe Ser His Val Thr Phe Pro Gly Thr Thr Ala Val 115 120 125
Thr Leu Ser Gly Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly 130 135 140
Ile Ser Arg Thr Gly Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser 145 150 155 160
Tyr Leu Asp Leu Met Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val 165 170 175
Ala Arg Ala Met Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg 180 185 190
Phe Arg Gln Ile Gln Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser 195 200 205
Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn 210 215 220
Trp Gly Arg Leu Ser Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser 225 230 235 240
Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly 245 250 255

Ser Val Ala Leu Ile Leu Asn Cys His His His Ala Ser Arg Val Ala
 260 265 270
 Arg Met Ala Ser Asp Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg
 275 280 285
 Val Arg Gly Ile Thr His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu
 290 295 300
 Gly Ala Ile Leu Met Arg Arg Thr Ile Ser Ser Leu Glu His His His
 305 310 315 320
 His His His

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 294 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..294

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATG AAA AAA ACA TTA TTA ATA GCT GCA TCG CTT TCA TTT TTT TCA GCA	48
Met Lys Lys Thr Leu Leu Ile Ala Ala Ser Leu Ser Phe Phe Ser Ala	
1 5 10 15	
AGT GCG CTG GCG ACG CCT GAT TGT GTA ACT GGA AAG GTG GAG TAT ACA	96
Ser Ala Leu Ala Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr	
20 25 30	
AAA TAT AAT GAT GAC GAT ACC TTT ACA GTT AAA GTG GGT GAT AAA GAA	144
Lys Tyr Asn Asp Asp Asp Thr Phe Thr Val Lys Val Gly Asp Lys Glu	
35 40 45	
TTA TTT ACC AAC AGA TGG AAT CTT CAG TCT CTT CTT CTC AGT GCG CAA	192
Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu Ser Ala Gln	
50 55 60	
ATT ACG GGG ATG ACT GTA ACC ATT AAA ACT AAT GCC TGT CAT AAT GGA	240
Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly	
65 70 75 80	
GGG GGA TTC AGC GAA GTT ATT TTT CGT CTC GAG CAC CAC CAC CAC	288
Gly Gly Phe Ser Glu Val Ile Phe Arg Leu Glu His His His His His	
85 90 95	
CAC TG	294
His	

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 97 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Met Lys Lys Thr Leu Leu Ile Ala Ala Ser Leu Ser Phe Phe Ser Ala

1	5	10	15													
Ser	Ala	Leu	Ala	Thr	Pro	Asp	Cys	Val	Thr	Gly	Lys	Val	Glu	Tyr	Thr	
		20						25					30			
Lys	Tyr	Asn	Asp	Asp	Asp	Asp	Thr	Phe	Thr	Val	Lys	Val	Gly	Asp	Lys	Glu
		35						40			45					
Leu	Phe	Thr	Asn	Arg	Trp	Asn	Leu	Gln	Ser	Leu	Leu	Leu	Ser	Ala	Gln	
		50					55			60						
Ile	Thr	Gly	Met	Thr	Val	Thr	Ile	Lys	Thr	Asn	Ala	Cys	His	Asn	Gly	
		65			70			75			80					
Gly	Gly	Phe	Ser	Glu	Val	Ile	Phe	Arg	Leu	Glu	His	His	His	His	His	
		85						90			95					

His

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 981 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..981

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

ATG AAG TGT ATA TTA TTT AAA TGG GTA CTG TGC CTG TTA CTG GGT TTT	48
Met Lys Cys Ile Leu Phe Lys Trp Val Leu Cys Leu Leu Leu Gly Phe	
1 5 10 15	
TCT TCG GTA TCC TAT TCC CGG GAG TTT ACG ATA GAC TTT TCG ACC CAA	96
Ser Ser Val Ser Tyr Ser Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln	
20 25 30	
CAA AGT TAT GTC TCT TCG TTA AAT AGT ATA CGG ACA GAG ATA TCG ACC	144
Gln Ser Tyr Val Ser Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr	
35 40 45	
CCT CTT GAA CAT ATA TCT CAG GGG ACC ACA TCG GTG TCT GTT ATT AAC	192
Pro Leu Glu His Ile Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn	
50 55 60	
CAC ACC CAC GGC AGT TAT TTT GCT GTG GAT ATA CGA GGG CTT GAT GTC	240
His Thr His Gly Ser Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val	
65 70 75 80	
TAT CAG GCG CGT TTT GAC CAT CTT CGT CTG ATT ATT GAG CAA AAT AAT	288
Tyr Gln Ala Arg Phe Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn	
85 90 95	
TTA TAT GTG GCA GGG TTC GTT AAT ACG GCA ACA AAT ACT TTC TAC CGT	336
Leu Tyr Val Ala Gly Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg	
100 105 110	
TTT TCA GAT TTT ACA CAT ATA TCA GTG CCC GGT GTG ACA ACG GTT TCC	384
Phe Ser Asp Phe Thr His Ile Ser Val Pro Gly Val Thr Thr Val Ser	
115 120 125	
ATG ACA ACG GAC AGC AGT TAT ACC ACT CTG CAA CGT GTC GCA GCG CTG	432
Met Thr Thr Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu	
130 135 140	

GAA CGT TCC GGA ATG CAA ATC AGT CGT CAC TCA CTG GTT TCA TCA TAT Glu Arg Ser Gly Met Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr 145 150 155 160	480
CTG GCG TTA ATG GAG TTC AGT GGT AAT ACA ATG ACC AGA GAT GCA TCC Leu Ala Leu Met Glu Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser 165 170 175	528
AGA GCA GTT CTG CGT TTT GTC ACT GTC ACA GCA GAA GCC TTA CGC TTC Arg Ala Val Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe 180 185 190	576
AGG CAG ATA CAG AGA GAA TTT CGT CAG GCA CTG TCT GAA ACT GCT CCT Arg Gln Ile Gln Arg Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro 195 200 205	624
GTG TAT ACG ATG ACG CCG GGA GAC GTG GAC CTC ACT CTG AAC TGG GGG Val Tyr Thr Met Thr Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly 210 215 220	672
CGA ATC AGC AAT GTG CTT CCG GAG TAT CGG GGA GAG GAT GGT GTC AGA Arg Ile Ser Asn Val Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg 225 230 235 240	720
GTG GGG AGA ATA TCC TTT AAT AAT ATA TCA GCG ATA CTG GGG ACT GTG Val Gly Arg Ile Ser Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val 245 250 255	768
GCC GTT ATA CTG AAT TGC CAT CAT CAG GGG GCG CGT TCT GTT CGC GCC Ala Val Ile Leu Asn Cys His His Gln Gly Ala Arg Ser Val Arg Ala 260 265 270	816
GTG AAT GAA GAG AGT CAA CCA GAA TGT CAG ATA ACT GGC GAC AGG CCT Val Asn Glu Glu Ser Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro 275 280 285	864
GTT ATA AAA ATA AAC AAT ACA TTA TGG GAA AGT AAT ACA GCT GCA GCG Val Ile Lys Ile Asn Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala 290 295 300	912
TTT CTG AAC AGA AAG TCA CAG TTT TTA TAT ACA ACG GGT AAA CTC GAG Phe Leu Asn Arg Lys Ser Gln Phe Leu Tyr Thr Thr Gly Lys Leu Glu 305 310 315 320	960
CAC CAC CAC CAC CAC CAC TG His His His His His His 325	981

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 326 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Met Lys Cys Ile Leu Phe Lys Trp Val Leu Cys Leu Leu Leu Gly Phe 1 5 10 15
Ser Ser Val Ser Tyr Ser Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln 20 25 30
Gln Ser Tyr Val Ser Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr 35 40 45
Pro Leu Glu His Ile Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn 50 55 60

His Thr His Gly Ser Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val
 65 70 75 80
 Tyr Gln Ala Arg Phe Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn
 85 90 95
 Leu Tyr Val Ala Gly Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg
 100 105 110
 Phe Ser Asp Phe Thr His Ile Ser Val Pro Gly Val Thr Thr Val Ser
 115 120 125
 Met Thr Thr Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu
 130 135 140
 Glu Arg Ser Gly Met Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr
 145 150 155 160
 Leu Ala Leu Met Glu Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser
 165 170 175
 Arg Ala Val Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe
 180 185 190
 Arg Gln Ile Gln Arg Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro
 195 200 205
 Val Tyr Thr Met Thr Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly
 210 215 220
 Arg Ile Ser Asn Val Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg
 225 230 235 240
 Val Gly Arg Ile Ser Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val
 245 250 255
 Ala Val Ile Leu Asn Cys His His Gln Gly Ala Arg Ser Val Arg Ala
 260 265 270
 Val Asn Glu Glu Ser Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro
 275 280 285
 Val Ile Lys Ile Asn Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala
 290 295 300
 Phe Leu Asn Arg Lys Ser Gln Phe Leu Tyr Thr Thr Gly Lys Leu Glu
 305 310 315 320
 His His His His His
 325

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 294 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..294

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ATG AAG AAG ATG TTT ATG GCG GTT TTA TTT GCA TTA GCT TCT GTT AAT
 Met Lys Lys Met Phe Met Ala Val Leu Phe Ala Leu Ala Ser Val Asn
 1 5 10 15

48

GCA ATG GCG GCG GAT TGT GCT AAA GGT AAA ATT GAG TTT TCC AAG TAT Ala Met Ala Ala Asp Cys Ala Lys Gly Lys Ile Glu Phe Ser Lys Tyr 20 25 30	96
AAT GAG GAT GAC ACA TTT ACA GTG AAG GTT GAC GGG AAA GAA TAC TGG Asn Glu Asp Asp Thr Phe Thr Val Lys Val Asp Gly Lys Glu Tyr Trp 35 40 45	144
ACC AGT CGC TGG AAT CTG CAA CCG TTA CTG CAA AGT GCT CAG TTG ACA Thr Ser Arg Trp Asn Leu Gln Pro Leu Leu Gln Ser Ala Gln Leu Thr 50 55 60	192
GGA ATG ACT GTC ACA ATC AAA TCC AGT ACC TGT GAA TCA GGC TCC GGA Gly Met Thr Val Thr Ile Lys Ser Ser Thr Cys Glu Ser Gly Ser Gly 65 70 75 80	240
TTT GCT GAA GTG CAG TTT AAT AAT GAC CTC GAG CAC CAC CAC CAC Phe Ala Glu Val Gln Phe Asn Asn Asp Leu Glu His His His His His 85 90 95	288
CAC TG His	294

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 97 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Met Lys Lys Met Phe Met Ala Val Leu Phe Ala Leu Ala Ser Val Asn 1 5 10 15
--

Ala Met Ala Ala Asp Cys Ala Lys Gly Lys Ile Glu Phe Ser Lys Tyr 20 25 30

Asn Glu Asp Asp Thr Phe Thr Val Lys Val Asp Gly Lys Glu Tyr Trp 35 40 45

Thr Ser Arg Trp Asn Leu Gln Pro Leu Leu Gln Ser Ala Gln Leu Thr 50 55 60

Gly Met Thr Val Thr Ile Lys Ser Ser Thr Cys Glu Ser Gly Ser Gly 65 70 75 80
--

Phe Ala Glu Val Gln Phe Asn Asn Asp Leu Glu His His His His His 85 90 95

His

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CGGAATTCAA GGAATTACCG TTAGACTTCT CG

32

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GGCTCGAGTC AACTGCTAAT AGTTCTGC

28

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CGGAATTCCG GGAGTTTACG ATAGACTTTT CG

32

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GGCTCGAGTT ATTTACCCGT TGTATATAA

29

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2127 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..2127
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

ATG AAA ATA AAA ACA GGT GCA CGC ATC CTC GCA TTA TCC GCA TTA ACG	48		
Met Lys Ile Lys Thr Gly Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr			
1	5	10	15

ACG ATG ATG TTT TCC GCC TCG GCT CTC GCC AAA ATC GAA GAA GGT AAA	96	
Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys		
20	25	30

CTG GTA ATC TGG ATT AAC GGC GAT AAA GGC TAT AAC GGT CTC GCT GAA	144	
Leu Val Ile Trp Ile Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu		
35	40	45

GTC GGT AAG AAA TTC GAG AAA GAT ACC GGA ATT AAA GTC ACC GTT GAG	192
Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu	

50	55	60	
CAT CCG GAT AAA CTG GAA GAG AAA TTC CCA CAG GTT GCG GCA ACT GGC His Pro Asp Lys Leu Glu Glu Lys Phe Pro Gln Val Ala Ala Thr Gly 65 70 75 80			240
GAT GGC CCT GAC ATT ATC TTC TGG GCA CAC GAC CGC TTT GGT GGC TAC Asp Gly Pro Asp Ile Ile Phe Trp Ala His Asp Arg Phe Gly Gly Tyr 85 90 95			288
GCT CAA TCT GGC CTG TTG GCT GAA ATC ACC CCG GAC AAA GCG TTC CAG Ala Gln Ser Gly Leu Leu Ala Glu Ile Thr Pro Asp Lys Ala Phe Gln 100 105 110			336
GAC AAG CTG TAT CCG TTT ACC TGG GAT GCC GTA CGT TAC AAC GGC AAG Asp Lys Leu Tyr Pro Phe Thr Trp Asp Ala Val Arg Tyr Asn Gly Lys 115 120 125			384
CTG ATT GCT TAC CCG ATC GCT GTT GAA GCG TTA TCG CTG ATT TAT AAC Leu Ile Ala Tyr Pro Ile Ala Val Glu Ala Leu Ser Leu Ile Tyr Asn 130 135 140			432
AAA GAT CTG CTG CCG AAC CCG CCA AAA ACC TGG GAA GAG ATC CCG GCG Lys Asp Leu Leu Pro Asn Pro Pro Lys Thr Trp Glu Glu Ile Pro Ala 145 150 155 160			480
CTG GAT AAA GAA CTG AAA GCG AAA GGT AAG AGC GCG CTG ATG TTC AAC Leu Asp Lys Glu Leu Lys Ala Lys Gly Ser Ala Leu Met Phe Asn 165 170 175			528
CTG CAA GAA CCG TAC TTC ACC TGG CCG CTG ATT GCT GCT GAC GGG GGT Leu Gln Glu Pro Tyr Phe Thr Trp Pro Leu Ile Ala Ala Asp Gly Gly 180 185 190			576
TAT GCG TTC AAG TAT GAA AAC GGC AAG TAC GAC ATT AAA GAC GTG GGC Tyr Ala Phe Lys Tyr Glu Asn Gly Lys Tyr Asp Ile Lys Asp Val Gly 195 200 205			624
GTG GAT AAC GCT GGC GCG AAA GCG GGT CTG ACC TTC CTG GTT GAC CTG Val Asp Asn Ala Gly Ala Lys Ala Gly Leu Thr Phe Leu Val Asp Leu 210 215 220			672
ATT AAA AAC AAA CAC ATG AAT GCA GAC ACC GAT TAC TCC ATC GCA GAA Ile Lys Asn Lys His Met Asn Ala Asp Thr Asp Tyr Ser Ile Ala Glu 225 230 235 240			720
GCT GCC TTT AAT AAA GGC GAA ACA GCG ATG ACC ATC AAC GGC CCG TGG Ala Ala Phe Asn Lys Gly Glu Thr Ala Met Thr Ile Asn Gly Pro Trp 245 250 255			768
GCA TGG TCC AAC ATC GAC ACC AGC AAA GTG AAT TAT GGT GTA ACG GTA Ala Trp Ser Asn Ile Asp Thr Ser Lys Val Asn Tyr Gly Val Thr Val 260 265 270			816
CTG CCG ACC TTC AAG GGT CAA CCA TCC AAA CCG TTC GTT GGC GTG CTG Leu Pro Thr Phe Lys Gly Gln Pro Ser Lys Pro Phe Val Gly Val Leu 275 280 285			864
AGC GCA GGT ATT AAC GCC GCC AGT CCG AAC AAA GAG CTG GCG AAA GAG Ser Ala Gly Ile Asn Ala Ala Ser Pro Asn Lys Glu Leu Ala Lys Glu 290 295 300			912

TTC CTC GAA AAC TAT CTG CTG ACT GAT GAA GGT CTG GAA GCG GTT AAT Phe Leu Glu Asn Tyr Leu Leu Thr Asp Glu Gly Leu Glu Ala Val Asn 305 310 315 320	960
AAA GAC AAA CCG CTG GGT GCC GTA GCG CTG AAG TCT TAC GAG GAA GAG Lys Asp Lys Pro Leu Gly Ala Val Ala Leu Lys Ser Tyr Glu Glu Glu 325 330 335	1008
TTG GCG AAA GAT CCA CGT ATT GCC GCC ACC ATG GAA AAC GCC CAG AAA Leu Ala Lys Asp Pro Arg Ile Ala Ala Thr Met Glu Asn Ala Gln Lys 340 345 350	1056
GGT GAA ATC ATG CCG AAC ATC CCG CAG ATG TCC GCT TTC TGG TAT GCC Gly Glu Ile Met Pro Asn Ile Pro Gln Met Ser Ala Phe Trp Tyr Ala 355 360 365	1104
GTG CGT ACT GCG GTG ATC AAC GCC GCC AGC GGT CGT CAG ACT GTC GAT Val Arg Thr Ala Val Ile Asn Ala Ala Ser Gly Arg Gln Thr Val Asp 370 375 380	1152
GAA GCC CTG AAA GAC GCG CAG ACT TCG AGC TCG AAC AAC AAC AAC AAT Glu Ala Leu Lys Asp Ala Gln Thr Ser Ser Ser Asn Asn Asn Asn Asn 385 390 395 400	1200
AAC AAT AAC AAC AAC CTC GGG ATC GAG GGA AGG ATT TCA GAA TTC AAG Asn Asn Asn Asn Leu Gly Ile Glu Gly Arg Ile Ser Glu Phe Lys 405 410 415	1248
GAA TTT ACC TTA GAC TTC TCG ACT GCA AAG ACG TAT GTA GAT TCG CTG Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp Ser Leu 420 425 430	1296
AAT GTC ATT CGC TCT GCA ATA GGT ACT CCA TTA CAG ACT ATT TCA TCA Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile Ser Ser 435 440 445	1344
GGA GGT ACG TCT TTA CTG ATG ATT GAT AGT GGC TCA GGG GAT AAT TTG Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Ser Gly Asp Asn Leu 450 455 460	1392
TTT GCA GTT GAT GTC AGA GGG ATA GAT GCA GAG GAA GGG CGG TTT AAT Phe Ala Val Asp Val Arg Gly Ile Asp Ala Glu Glu Gly Arg Phe Asn 465 470 475 480	1440
AAT CTA CGG CTT ATT GTT GAA CGA AAT AAT TTA TAT GTG ACA GGA TTT Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr Gly Phe 485 490 495	1488
GTT AAC AGG ACA AAT AAT GTT TTT TAT CGC TTT GCT GAT TTT TCA CAT Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe Ser His 500 505 510	1536
GTT ACC TTT CCA GGT ACA ACA GCG GTT ACA TTG TCT GGT GAC AGT AGC Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp Ser Ser 515 520 525	1584
TAT ACC ACG TTA CAG CGT GTT GCA GGG ATC AGT CGT ACG GGG ATG CAG Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly Met Gln 530 535 540	1632
ATA AAT CGC CAT TCG TTG ACT ACT TCT TAT CTG GAT TTA ATG TCG CAT Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met Ser His 545 550 555 560	1680
AGT GGA ACC TCA CTG ACG CAG TCT GTG GCA AGA GCG ATG TTA CGG TTT Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu Arg Phe 565 570 575	1728
GTT ACT GTG ACA GCT GAA GCT TTA CGT TTT CGG CAA ATA CAG AGG GGA Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Gly	1776

580	585	590	
TTT CGT ACA ACA CTG GAT GAT CTC AGT GGG CGT TCT TAT GTA ATG ACT Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val Met Thr 595	600	605	1824
GCT GAA GAT GTT GAT CTT ACA TTG AAC TGG GGA AGG TTG AGT AGC GTC Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser Ser Val 610	615	620	1872
CTG CCT GAC TAT CAT GGA CAA GAC TCT GTT CGT GTA GGA AGA ATT TCT Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg Ile Ser 625	630	635	1920
TTT GGA AGC ATT AAT GCA ATT CTG GGA AGC GTG GCA TTA ATA CTG AAT Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser-Val Ala Leu Ile Leu Asn 645	650	655	1968
TGT CAT CAT CAT GCA TCG CGA GTT GCC AGA ATG GCA TCT GAT GAG TTT Cys His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp Glu Phe 660	665	670	2016
CCT TCT ATG TGT CCG GCA GAT GGA AGA GTC CGT GGG ATT ACG CAC AAT Pro Ser Met Cys Pro Ala Asp Gly Arg Val Arg Gly Ile Thr His Asn 675	680	685	2064
AAA ATA TTG TGG GAT TCA TCC ACT CTG GGG GCA ATT CTG ATG CGC AGA Lys Ile Leu Trp Asp Ser Ser Thr Leu Gly Ala Ile Leu Met Arg Arg 690	695	700	2112
ACT ATT AGC AGT TG Thr Ile Ser Ser 705			2127

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 708 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Met	Lys	Ile	Lys	Thr	Gly	Ala	Arg	Ile	Leu	Ala	Leu	Ser	Ala	Leu	Thr
1					5				10					15	
Thr	Met	Met	Phe	Ser	Ala	Ser	Ala	Leu	Ala	Lys	Ile	Glu	Glu	Gly	Lys
					20			25		30					
Leu	Val	Ile	Trp	Ile	Asn	Gly	Asp	Lys	Gly	Tyr	Asn	Gly	Leu	Ala	Glu
					35		40			45					
Val	Gly	Lys	Phe	Glu	Lys	Asp	Thr	Gly	Ile	Lys	Val	Thr	Val	Glu	
	50			55					60						
His	Pro	Asp	Lys	Leu	Glu	Glu	Lys	Phe	Pro	Gln	Val	Ala	Ala	Thr	Gly
	65			70				75		80					
Asp	Gly	Pro	Asp	Ile	Ile	Phe	Trp	Ala	His	Asp	Arg	Phe	Gly	Tyr	
	85						90				95				
Ala	Gln	Ser	Gly	Leu	Leu	Ala	Glu	Ile	Thr	Pro	Asp	Lys	Ala	Phe	Gln
	100						105				110				
Asp	Lys	Leu	Tyr	Pro	Phe	Thr	Trp	Asp	Ala	Val	Arg	Tyr	Asn	Gly	Lys
	115						120				125				
Leu	Ile	Ala	Tyr	Pro	Ile	Ala	Val	Glu	Ala	Leu	Ser	Leu	Ile	Tyr	Asn

130	135	140
Lys Asp Leu Leu Pro Asn Pro Pro Lys Thr Trp Glu Glu Ile Pro Ala		
145 150 155 160		
Leu Asp Lys Glu Leu Lys Ala Lys Gly Lys Ser Ala Leu Met Phe Asn		
165 170 175		
Leu Gln Glu Pro Tyr Phe Thr Trp Pro Leu Ile Ala Ala Asp Gly Gly		
180 185 190		
Tyr Ala Phe Lys Tyr Glu Asn Gly Lys Tyr Asp Ile Lys Asp Val Gly		
195 200 205		
Val Asp Asn Ala Gly Ala Lys Ala Gly Leu Thr Phe Leu Val Asp Leu		
210 215 220		
Ile Lys Asn Lys His Met Asn Ala Asp Thr Asp Tyr Ser Ile Ala Glu		
225 230 235 240		
Ala Ala Phe Asn Lys Gly Glu Thr Ala Met Thr Ile Asn Gly Pro Trp		
245 250 255		
Ala Trp Ser Asn Ile Asp Thr Ser Lys Val Asn Tyr Gly Val Thr Val		
260 265 270		
Leu Pro Thr Phe Lys Gly Gln Pro Ser Lys Pro Phe Val Gly Val Leu		
275 280 285		
Ser Ala Gly Ile Asn Ala Ala Ser Pro Asn Lys Glu Leu Ala Lys Glu		
290 295 300		
Phe Leu Glu Asn Tyr Leu Leu Thr Asp Glu Gly Leu Glu Ala Val Asn		
305 310 315 320		
Lys Asp Lys Pro Leu Gly Ala Val Ala Leu Lys Ser Tyr Glu Glu Glu		
325 330 335		
Leu Ala Lys Asp Pro Arg Ile Ala Ala Thr Met Glu Asn Ala Gln Lys		
340 345 350		
Gly Glu Ile Met Pro Asn Ile Pro Gln Met Ser Ala Phe Trp Tyr Ala		
355 360 365		
Val Arg Thr Ala Val Ile Asn Ala Ala Ser Gly Arg Gln Thr Val Asp		
370 375 380		
Glu Ala Leu Lys Asp Ala Gln Thr Ser Ser Ser Asn Asn Asn Asn Asn		
385 390 395 400		
Asn Asn Asn Asn Leu Gly Ile Glu Gly Arg Ile Ser Glu Phe Lys		
405 410 415		
Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp Ser Leu		
420 425 430		
Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile Ser Ser		
435 440 445		
Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Ser Gly Asp Asn Leu		
450 455 460		
Phe Ala Val Asp Val Arg Gly Ile Asp Ala Glu Glu Gly Arg Phe Asn		
465 470 475 480		
Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr Gly Phe		
485 490 495		
Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe Ser His		
500 505 510		

Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp Ser Ser
 515 520 525
 Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly Met Gln
 530 535 540
 Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met Ser His
 545 550 555 560
 Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu Arg Phe
 565 570 575
 Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Gly
 580 585 590
 Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val Met Thr
 595 600 605
 Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser Ser Val
 610 615 620
 Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg Ile Ser
 625 630 635 640
 Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile Leu Asn
 645 650 655
 Cys His His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp Glu Phe
 660 665 670
 Pro Ser Met Cys Pro Ala Asp Gly Arg Val Arg Gly Ile Thr His Asn
 675 680 685
 Lys Ile Leu Trp Asp Ser Ser Thr Leu Gly Ala Ile Leu Met Arg Arg
 690 695 700
 Thr Ile Ser Ser
 705

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 2136 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 1..2136

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

ATG AAA ATA AAA ACA GGT GCA CGC ATC CTC GCA TTA TCC GCA TTA ACG	48
Met Lys Ile Lys Thr Gly Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr	
1 5 10 15	
ACG ATG ATG TTT TCC GCC TCG GCT CTC GCC AAA ATC GAA GAA GGT AAA	96
Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys	
20 25 30	
CTG GTA ATC TGG ATT AAC GGC GAT AAA GGC TAT AAC GGT CTC GCT GAA	144
Leu Val Ile Trp Ile Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu	
35 40 45	
GTC GGT AAG AAA TTC GAG AAA GAT ACC GGA ATT AAA GTC ACC GTT GAG	192
Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu	
50 55 60	

CAT CCG GAT AAA CTG GAA GAG AAA TTC CCA CAG GTT GCG GCA ACT GGC His Pro Asp Lys Leu Glu Glu Lys Phe Pro Gin Val Ala Ala Thr Gly 65 70 75 80	240
GAT GGC CCT GAC ATT ATC TTC TGG GCA CAC GAC CGC TTT GGT GGC TAC Asp Gly Pro Asp Ile Ile Phe Trp Ala His Asp Arg Phe Gly Gly Tyr 85 90 95	288
GCT CAA TCT GGC CTG TTG GCT GAA ATC ACC CCG GAC AAA GCG TTC CAG Ala Gln Ser Gly Leu Leu Ala Glu Ile Thr Pro Asp Lys Ala Phe Gln 100 105 110	336
GAC AAG CTG TAT CCG TTT ACC TGG GAT GCC GTA CGT TAC AAC GGC AAG Asp Lys Leu Tyr Pro Phe Thr Trp Asp Ala Val Arg Tyr Asn Gly Lys 115 120 125	384
CTG ATT GCT TAC CCG ATC GCT GTT GAA GCG TTA TCG CTG ATT TAT AAC Leu Ile Ala Tyr Pro Ile Ala Val Glu Ala Leu Ser Leu Ile Tyr Asn 130 135 140	432
AAA GAT CTG CTG CCG AAC CCG CCA AAA ACC TGG GAA GAG ATC CCG GCG Lys Asp Leu Leu Pro Asn Pro Lys Thr Trp Glu Glu Ile Pro Ala 145 150 155 160	480
CTG GAT AAA GAA CTG AAA GCG AAA GGT AAG AGC GCG CTG ATG TTC AAC Leu Asp Lys Glu Leu Lys Ala Lys Gly Lys Ser Ala Leu Met Phe Asn 165 170 175	528
CTG CAA GAA CCG TAC TTC ACC TGG CCG CTG ATT GCT GCT GAC GGG GGT Leu Gln Glu Pro Tyr Phe Thr Trp Pro Leu Ile Ala Ala Asp Gly Gly 180 185 190	576
TAT GCG TTC AAG TAT GAA AAC GGC AAG TAC GAC ATT AAA GAC GTG GGC Tyr Ala Phe Lys Tyr Glu Asn Gly Lys Tyr Asp Ile Lys Asp Val Gly 195 200 205	624
GTG GAT AAC GCT GGC GCG AAA GCG GGT CTG ACC TTC CTG GTT GAC CTG Val Asp Asn Ala Gly Ala Lys Ala Gly Leu Thr Phe Leu Val Asp Leu 210 215 220	672
ATT AAA AAC AAA CAC ATG AAT GCA GAC ACC GAT TAC TCC ATC GCA GAA Ile Lys Asn Lys His Met Asn Ala Asp Thr Asp Tyr Ser Ile Ala Glu 225 230 235 240	720
GCT GCC TTT AAT AAA GGC GAA ACA GCG ATG ACC ATC AAC GGC CCG TGG Ala Ala Phe Asn Lys Gly Glu Thr Ala Met Thr Ile Asn Gly Pro Trp 245 250 255	768
GCA TGG TCC AAC ATC GAC ACC AGC AAA GTG AAT TAT GGT GTA ACG GTA Ala Trp Ser Asn Ile Asp Thr Ser Lys Val Asn Tyr Gly Val Thr Val 260 265 270	816
CTG CCG ACC TTC AAG GGT CAA CCA TCC AAA CCG TTC GTT GGC GTG CTG Leu Pro Thr Phe Lys Gly Gln Pro Ser Lys Pro Phe Val Gly Val Leu 275 280 285	864
AGC GCA GGT ATT AAC GCC GCC AGT CCG AAC AAA GAG CTG GCG AAA GAG Ser Ala Gly Ile Asn Ala Ala Ser Pro Asn Lys Glu Leu Ala Lys Glu 290 295 300	912
TTC CTC GAA AAC TAT CTG CTG ACT GAT GAA GGT CTG GAA GCG GTT AAT Phe Leu Glu Asn Tyr Leu Leu Thr Asp Glu Gly Leu Glu Ala Val Asn 305 310 315 320	960
AAA GAC AAA CCG CTG GGT GCC GTA GCG CTG AAG TCT TAC GAG GAA GAG Lys Asp Lys Pro Leu Gly Ala Val Ala Leu Lys Ser Tyr Glu Glu Glu 325 330 335	1008
TTG GCG AAA GAT CCA CGT ATT GCC GCC ACC ATG GAA AAC GCC CAG AAA Leu Ala Lys Asp Pro Arg Ile Ala Ala Thr Met Glu Asn Ala Gln Lys	1056

340	345	350	
GGT GAA ATC ATG CCG AAC ATC CCG CAG ATG TCC GCT TTC TGG TAT GCC Gly Glu Ile Met Pro Asn Ile Pro Gln Met Ser Ala Phe Trp Tyr Ala 355	360	365	1104
GTG CGT ACT GCG GTG ATC AAC GCC GCC AGC GGT CGT CAG ACT GTC GAT Val Arg Thr Ala Val Ile Asn Ala Ala Ser Gly Arg Gln Thr Val Asp 370	375	380	1152
GAA GCC CTG AAA GAC GCG CAG ACT TCG AGC TCG AAC AAC AAC AAC AAT Glu Ala Leu Lys Asp Ala Gln Thr Ser Ser Asn Asn Asn Asn Asn 385	390	395	1200
AAC AAT AAC AAC AAC CTC GGG ATC GAG GGA AGG ATT TCA GAA TTC CGG Asn Asn Asn Asn Leu Gly Ile Glu Gly Arg Ile Ser Glu Phe Arg 405	410	415	1248
GAG TTT ACG ATA GAC TTT TCG ACC CAA CAA AGT TAT GTC TCT TCG TTA Glu Phe Thr Ile Asp Phe Ser Thr Gln Gln Ser Tyr Val Ser Ser Leu 420	425	430	1296
AAT AGT ATA CGG ACA GAG ATA TCG ACC CCT CTT GAA CAT ATA TCT CAG Asn Ser Ile Arg Thr Glu Ile Ser Thr Pro Leu Glu His Ile Ser Gln 435	440	445	1344
GGG ACC ACA TCG GTG TCT GTT ATT AAC CAC ACC CAC GGC AGT TAT TTT Gly Thr Thr Ser Val Ser Val Ile Asn His Thr His Gly Ser Tyr Phe 450	455	460	1392
GCT GTG GAT ATA CGA GGG CTT GAT GTC TAT CAG GCG CGT TTT GAC CAT Ala Val Asp Ile Arg Gly Leu Asp Val Tyr Gln Ala Arg Phe Asp His 465	470	475	1440
CTT CGT CTG ATT ATT GAG CAA AAT AAT TTA TAT GTG GCA GGG TTC GTT Leu Arg Leu Ile Ile Glu Gln Asn Asn Leu Tyr Val Ala Gly Phe Val 485	490	495	1488
AAT ACG GCA ACA AAT ACT TTC TAC CGT TTT TCA GAT TTT ACA CAT ATA Asn Thr Ala Thr Asn Thr Phe Tyr Arg Phe Ser Asp Phe Thr His Ile 500	505	510	1536
TCA GTG CCC GGT GTG ACA ACG GTT TCC ATG ACA ACG GAC AGC AGT TAT Ser Val Pro Gly Val Thr Thr Val Ser Met Thr Thr Asp Ser Ser Tyr 515	520	525	1584
ACC ACT CTG CAA CGT GTC GCA GCG CTG GAA CGT TCC GGA ATG CAA ATC Thr Thr Leu Gln Arg Val Ala Ala Leu Glu Arg Ser Gly Met Gln Ile 530	535	540	1632
AGT CGT CAC TCA CTG GTT TCA TCA TAT CTG GCG TTA ATG GAG TTC AGT Ser Arg His Ser Leu Val Ser Ser Tyr Leu Ala Leu Met Glu Phe Ser 545	550	555	1680
GGT AAT ACA ATG ACC AGA GAT GCA TCC AGA GCA GTT CTG CGT TTT GTC Gly Asn Thr Met Thr Arg Asp Ala Ser Arg Ala Val Leu Arg Phe Val 565	570	575	1728
ACT GTC ACA GCA GAA GCC TTA CGC TTC AGG CAG ATA CAG AGA GAA TTT Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Glu Phe 580	585	590	1776
CGT CAG GCA CTG TCT GAA ACT GCT CCT GTG TAT ACG ATG ACG CCG GGA Arg Gln Ala Leu Ser Glu Thr Ala Pro Val Tyr Thr Met Thr Pro Gly 595	600	605	1824
GAC GTG GAC CTC ACT CTG AAC TGG GGG CGA ATC AGC AAT GTG CTT CCG Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Ile Ser Asn Val Leu Pro 610	615	620	1872

GAG TAT CGG GGA GAG GAT GGT GTC AGA GTG GGG AGA ATA TCC TTT AAT Glu Tyr Arg Gly Glu Asp Gly Val Arg Val Gly Arg Ile Ser Phe Asn 625 630 635 640	1920
AAT ATA TCA GCG ATA CTG GGG ACT GTG GCC GTT ATA CTG AAT TGC CAT Asn Ile Ser Ala Ile Leu Gly Thr Val Ala Val Ile Leu Asn Cys His 645 650 655	1968
CAT CAG GGG GCG CGT TCT GTT CGC GCC GTG AAT GAA GAG AGT CAA CCA His Gln Gly Ala Arg Ser Val Arg Ala Val Asn Glu Ser Gln Pro 660 665 670	2016
GAA TGT CAG ATA ACT GGC GAC AGG CCT GTT ATA AAA ATA AAC AAT ACA Glu Cys Gln Ile Thr Gly Asp Arg Pro Val Ile Lys Ile Asn Asn Thr 675 680 685	2064
TTA TGG GAA AGT AAT ACA GCT GCA GCG TTT CTG AAC AGA AAG TCA CAG Leu Trp Glu Ser Asn Thr Ala Ala Ala Phe Leu Asn Arg Lys Ser Gln 690 695 700	2112
TTT TTA TAT ACA ACG GGT AAA TA Phe Leu Tyr Thr Gly Lys 705 710	2136

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 711 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Met Lys Ile Lys Thr Gly Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr 1 5 10 15
Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys 20 25 30
Leu Val Ile Trp Ile Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu 35 40 45
Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu 50 55 60
His Pro Asp Lys Leu Glu Lys Phe Pro Gln Val Ala Ala Thr Gly 65 70 75 80
Asp Gly Pro Asp Ile Ile Phe Trp Ala His Asp Arg Phe Gly Gly Tyr 85 90 95
Ala Gln Ser Gly Leu Leu Ala Glu Ile Thr Pro Asp Lys Ala Phe Gln 100 105 110
Asp Lys Leu Tyr Pro Phe Thr Trp Asp Ala Val Arg Tyr Asn Gly Lys 115 120 125
Leu Ile Ala Tyr Pro Ile Ala Val Glu Ala Leu Ser Leu Ile Tyr Asn 130 135 140
Lys Asp Leu Leu Pro Asn Pro Pro Lys Thr Trp Glu Glu Ile Pro Ala 145 150 155 160
Leu Asp Lys Glu Leu Lys Ala Lys Gly Lys Ser Ala Leu Met Phe Asn 165 170 175
Leu Gln Glu Pro Tyr Phe Thr Trp Pro Leu Ile Ala Ala Asp Gly Gly 180 185 190

Tyr Ala Phe Lys Tyr Glu Asn Gly Lys Tyr Asp Ile Lys Asp Val Gly
 195 200 205
 Val Asp Asn Ala Gly Ala Lys Ala Gly Leu Thr Phe Leu Val Asp Leu
 210 215 220
 Ile Lys Asn Lys His Met Asn Ala Asp Thr Asp Tyr Ser Ile Ala Glu
 225 230 235 240
 Ala Ala Phe Asn Lys Gly Glu Thr Ala Met Thr Ile Asn Gly Pro Trp
 245 250 255
 Ala Trp Ser Asn Ile Asp Thr Ser Lys Val Asn Tyr Gly Val Thr Val
 260 265 270
 Leu Pro Thr Phe Lys Gly Gln Pro Ser Lys Pro Phe Val Gly Val Leu
 275 280 285
 Ser Ala Gly Ile Asn Ala Ala Ser Pro Asn Lys Glu Leu Ala Lys Glu
 290 295 300
 Phe Leu Glu Asn Tyr Leu Leu Thr Asp Glu Gly Leu Glu Ala Val Asn
 305 310 315 320
 Lys Asp Lys Pro Leu Gly Ala Val Ala Leu Lys Ser Tyr Glu Glu
 325 330 335
 Leu Ala Lys Asp Pro Arg Ile Ala Ala Thr Met Glu Asn Ala Gln Lys
 340 345 350
 Gly Glu Ile Met Pro Asn Ile Pro Gln Met Ser Ala Phe Trp Tyr Ala
 355 360 365
 Val Arg Thr Ala Val Ile Asn Ala Ala Ser Gly Arg Gln Thr Val Asp
 370 375 380
 Glu Ala Leu Lys Asp Ala Gln Thr Ser Ser Ser Asn Asn Asn Asn Asn
 385 390 395 400
 Asn Asn Asn Asn Leu Gly Ile Glu Gly Arg Ile Ser Glu Phe Arg
 405 410 415
 Glu Phe Thr Ile Asp Phe Ser Thr Gln Gln Ser Tyr Val Ser Ser Leu
 420 425 430
 Asn Ser Ile Arg Thr Glu Ile Ser Thr Pro Leu Glu His Ile Ser Gln
 435 440 445
 Gly Thr Thr Ser Val Ser Val Ile Asn His Thr His Gly Ser Tyr Phe
 450 455 460
 Ala Val Asp Ile Arg Gly Leu Asp Val Tyr Gln Ala Arg Phe Asp His
 465 470 475 480
 Leu Arg Leu Ile Ile Glu Gln Asn Asn Leu Tyr Val Ala Gly Phe Val
 485 490 495
 Asn Thr Ala Thr Asn Thr Phe Tyr Arg Phe Ser Asp Phe Thr His Ile
 500 505 510
 Ser Val Pro Gly Val Thr Thr Val Ser Met Thr Thr Asp Ser Ser Tyr
 515 520 525
 Thr Thr Leu Gln Arg Val Ala Ala Leu Glu Arg Ser Gly Met Gln Ile
 530 535 540
 Ser Arg His Ser Leu Val Ser Ser Tyr Leu Ala Leu Met Glu Phe Ser
 545 550 555 560
 Gly Asn Thr Met Thr Arg Asp Ala Ser Arg Ala Val Leu Arg Phe Val

565	570	575
Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Glu Phe		
580	585	590
Arg Gln Ala Leu Ser Glu Thr Ala Pro Val Tyr Thr Met Thr Pro Gly		
595	600	605
Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Ile Ser Asn Val Leu Pro		
610	615	620
Glu Tyr Arg Gly Glu Asp Gly Val Arg Val Gly Arg Ile Ser Phe Asn		
625	630	635
Asn Ile Ser Ala Ile Leu Gly Thr Val Ala Val Ile Leu Asn Cys His		
645	650	655
His Gln Gly Ala Arg Ser Val Arg Ala Val Asn Glu Glu Ser Gln Pro		
660	665	670
Glu Cys Gln Ile Thr Gly Asp Arg Pro Val Ile Lys Ile Asn Asn Thr		
675	680	685
Leu Trp Glu Ser Asn Thr Ala Ala Ala Phe Leu Asn Arg Lys Ser Gln		
690	695	700
Phe Leu Tyr Thr Thr Gly Lys		
705	710	

(2) INFORMATION FOR SEQ ID NO:36:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 981 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..981
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT GGT TTC GCT	48
Met Lys Lys Thr Ala Ile Ala Ile Val Ala Leu Ala Gly Phe Ala	
1 5 10 15	
ACC GTT GCG CAA GCT GAC TAC AAG GAC GAC GAT GAC AAG AAG CTT GAA	96
Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Lys Leu Glu	
20 25 30	
TTC AAG GAA TTT ACC TTA GAC TTC TCG ACT GCA AAG ACG TAT GTA GAT	144
Phe Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp	
35 40 45	
TCG CTG AAT GTC ATT CGC TCT GCA ATA GGT ACT CCA TTA CAG ACT ATT	192
Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile	
50 55 60	
TCA TCA GGA GGT ACG TCT TTA CTG ATG ATT GAT AGT GGC TCA GGG GAT	240
Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Ser Gly Asp	
65 70 75 80	
AAT TTG TTT GCA GTT GAT GTC AGA GGG ATA GAT GCA GAG GAA GGG CGG	288
Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp Ala Glu Glu Gly Arg	
85 90 95	
TTT AAT AAT CTA CGG CTT ATT GTT GAA CGA AAT AAT TTA TAT GTG ACA	336

Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr	100	105	110	
GGA TTT GTT AAC AGG ACA AAT AAT GTT TTT TAT CGC TTT GCT GAT TTT				384
Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe	115	120	125	
TCA CAT GTT ACC TTT CCA GGT ACA ACA GCG GTT ACA TTG TCT GGT GAC				432
Ser His Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp	130	135	140	
AGT AGC TAT ACC ACG TTA CAG CGT GTT GCA GGG ATC AGT CGT ACG GGG				480
Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly	145	150	155	
ATG CAG ATA AAT CGC CAT TCG TTG ACT ACT TCT TAT CTG GAT TTA ATG				528
Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met	165	170	175	
TCG CAT AGT GGA ACC TCA CTG ACG CAG TCT GTG GCA AGA GCG ATG TTA				576
Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu	180	185	190	
CGG TTT GTT ACT GTG ACA GCT GAA GCT TTA CGT TTT CGG CAA ATA CAG				624
Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln	195	200	205	
AGG GGA TTT CGT ACA ACA CTG GAT GAT CTC AGT GGG CGT TCT TAT GCA				672
Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val	210	215	220	
ATG ACT GCT GAA GAT GTT GAT CTT ACA TTG AAC TGG GGA AGG TTG ACT				720
Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser	225	230	235	
AGC GTC CTG CCT GAC TAT CAT GGA CAA GAC TCT GTT CGT GTC GGA AGA				768
Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg	245	250	255	
ATT TCT TTT GGA AGC ATT AAT GCA ATT CTG GGA AGC GTG GCA TTA ATA				816
Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile	260	265	270	
CTG AAT TGT CAT CAT GCA TCG CGA GTT GCC AGA ATG GCA TCT GAT				864
Leu Asn Cys His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp	275	280	285	
GAG TTT CCT TCT ATG TGT CCG GCA GAT GGA AGA GTC CGT GGG ATT ACG				912
Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg Val Arg Gly Ile Thr	290	295	300	
CAC AAT AAA ATA TTG TGG GAT TCA TCC ACT CTG GGG GCA ATT CTG ATG				960
His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu Gly Ala Ile Leu Met	305	310	315	
CGC AGA ACT ATT AGC AGT TG				981
Arg Arg Thr Ile Ser Ser				
325				

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 326 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala
 1 5 10 15
 Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Asp Lys Lys Leu Glu
 20 25 30
 Phe Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp
 35 40 45
 Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile
 50 55 60
 Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Ser Gly Asp
 65 70 75 80
 Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp Ala Glu Glu Gly Arg
 85 90 95
 Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr
 100 105 110
 Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe
 115 120 125
 Ser His Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp
 130 135 140
 Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly
 145 150 155 160
 Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met
 165 170 175
 Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu
 180 185 190
 Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln
 195 200 205
 Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val
 210 215 220
 Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser
 225 230 235 240
 Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg
 245 250 255
 Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile
 260 265 270
 Leu Asn Cys His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp
 275 280 285
 Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg Val Arg Gly Ile Thr
 290 295 300
 His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu Gly Ala Ile Leu Met
 305 310 315 320
 Arg Arg Thr Ile Ser Ser
 325

(2) INFORMATION FOR SEQ ID NO:38:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 990 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..990

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT GGT TTC GCT	48
Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala	
1 5 10 15	
ACC GTT GCG CAA GCT GAC TAC AAG GAC GAT GAC AAG AAG CTT GAA	96
Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Asp Lys Lys Leu Glu	
20 25 30	
TTC CGG GAG TTT ACG ATA GAC TTT TCG ACC CAA CAA AGT TAT GTC TCT	144
Phe Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln Gln Ser Tyr Val Ser	
35 40 45	
TCG TTA AAT AGT ATA CGG ACA GAG ATA TCG ACC CCT CTT GAA CAT ATA	192
Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr Pro Leu Glu His Ile	
50 55 60	
TCT CAG GGG ACC ACA TCG GTG TCT GTT ATT AAC CAC ACC CAC GGC AGT	240
Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn His Thr His Gly Ser	
65 70 75 80	
TAT TTT GCT GTG GAT ATA CGA GGG CTT GAT GTC TAT CAG GCG CGT TTT	288
Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val Tyr Gln Ala Arg Phe	
85 90 95	
GAC CAT CTT CGT CTG ATT ATT GAG CAA AAT AAT TTA TAT GTG GCA GGG	336
Asp His Leu Arg Leu Ile Glu Gln Asn Asn Leu Tyr Val Ala Gly	
100 105 110	
TTC GTT AAT ACG GCA ACA AAT ACT TTC TAC CGT TTT TCA GAT TTT ACA	384
Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg Phe Ser Asp Phe Thr	
115 120 125	
CAT ATA TCA GTG CCC GGT GTG ACA ACG GTT TCC ATG ACA ACG GAC AGC	432
His Ile Ser Val Pro Gly Val Thr Thr Val Ser Met Thr Thr Asp Ser	
130 135 140	
AGT TAT ACC ACT CTG CAA CGT GTC GCA GCG CTG GAA CGT TCC GGA ATG	480
Ser Tyr Thr Leu Gln Arg Val Ala Ala Leu Glu Arg Ser Gly Met	
145 150 155 160	
CAA ATC AGT CGT CAC TCA CTG GTT TCA TCA TAT CTG GCG TTA ATG GAG	528
Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr Leu Ala Leu Met Glu	
165 170 175	
TTC AGT GGT AAT ACA ATG ACC AGA GAT GCA TCC AGA GCA GTT CTG CGT	576
Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser Arg Ala Val Leu Arg	
180 185 190	
TTT GTC ACT GTC ACA GCA GAA GCC TTA CGC TTC AGG CAG ATA CAG AGA	624
Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg	
195 200 205	
GAA TTT CGT CAG GCA CTG TCT GAA ACT GCT CCT GTG TAT ACG ATG ACG	672
Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro Val Tyr Thr Met Thr	
210 215 220	
CCG GGA GAC GTG GAC CTC ACT CTG AAC TGG GGG CGA ATC AGC AAT GTG	720
Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Ile Ser Asn Val	
225 230 235 240	
CTT CCG GAG TAT CGG GGA GAG GAT GGT GTC AGA GTG GGG AGA ATA TCC	768
Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg Val Gly Arg Ile Ser	

245	250	255	
TTT AAT AAT ATA TCA GCG ATA CTG GGG ACT GTG GCC GTT ATA CTG AAT Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val Ala Val Ile Leu Asn	260	265	816
TGC CAT CAT CAG GGG GCG CGT TCT GTT CGC GCC GTG AAT GAA GAG AGT Cys His His Gln Gly Ala Arg Ser Val Arg Ala Val Asn Glu Glu Ser	275	280	864
CAA CCA GAA TGT CAG ATA ACT GGC GAC AGG CCT GTT ATA AAA ATA AAC Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro Val Ile Lys Ile Asn	290	295	912
AAT ACA TTA TGG GAA AGT AAT ACA GCT GCA GCG TTT CTG AAC AGA AAG Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala Phe Leu Asn Arg Lys	305	310	960
TCA CAG TTT TTA TAT ACA ACG GGT AAA TA Ser Gln Phe Leu Tyr Thr Thr Gly Lys	325	330	990

(2) INFORMATION FOR SEQ ID NO:39:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 329 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala	1	5	10	15
Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Asp Lys Lys Leu Glu	20	25	30	
Phe Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln Gln Ser Tyr Val Ser	35	40	45	
Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr Pro Leu Glu His Ile	50	55	60	
Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn His Thr His Gly Ser	65	70	75	80
Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val Tyr Gln Ala Arg Phe	85	90	95	
Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn Leu Tyr Val Ala Gly	100	105	110	
Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg Phe Ser Asp Phe Thr	115	120	125	
His Ile Ser Val Pro Gly Val Thr Thr Val Ser Met Thr Thr Asp Ser	130	135	140	
Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu Glu Arg Ser Gly Met	145	150	155	160
Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr Leu Ala Leu Met Glu	165	170	175	
Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser Arg Ala Val Leu Arg	180	185	190	
Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg				

195

200

205

Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro Val Tyr Thr Met Thr
210 215 220

Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Ile Ser Asn Val
225 230 235 240

Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg Val Gly Arg Ile Ser
245 250 255

Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val Ala Val Ile Leu Asn
260 265 270

Cys His His Gln Gly Ala Arg Ser Val Arg Ala Val Asn Glu Glu Ser
275 280 285

Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro Val Ile Lys Ile Asn
290 295 300

Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala Phe Leu Asn Arg Lys
305 310 315 320

Ser Gln Phe Leu Tyr Thr Thr Gly Lys
325

CLAIMS

What is claimed is:

- 5 1. A method of treatment comprising:
 - a) providing:
 - i) antitoxin directed against at least a portion of an *Escherichia coli* verotoxin in an aqueous solution in therapeutic amount that is administrable, and
 - ii) an intoxicated subject; and
 - b) administering said antitoxin to said subject.
- 10 2. The method of Claim 1 wherein said *Escherichia coli* verotoxin is recombinant.
- 15 3. The method of Claim 1 wherein said antitoxin is an avian antitoxin.
- 20 4. The method of Claim 2 wherein said recombinant *Escherichia coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT1 sequence.
- 25 5. The method of Claim 2 wherein said recombinant *Escherichia coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT2 sequence.
- 30 6. The method of Claim 1 wherein said subject is an adult.
7. The method of Claim 1 wherein said subject is a child.
8. The method of Claim 1 wherein said administering is parenteral.
9. The method of Claim 1 wherein said administering is oral.

10. A method of prophylactic treatment comprising:

a) providing:

i) an antitoxin directed against at least one *Escherichia coli* verotoxin in an aqueous solution in therapeutic amount that is parenterally administrable, and

5 ii) at least one subject is at risk of diarrheal disease; and

b) parenterally administering said antitoxin to said subject.

11. The method of Claim 10, wherein said subject is at risk of developing extra-
10 intestinal complications of *Escherichia coli* infection.

12. The method of Claim 11, wherein said extra-intestinal complication is hemolytic uremic syndrome.

15 13. A composition comprising neutralizing antitoxin directed against at least one *Escherichia coli* verotoxin in an aqueous solution in therapeutic amounts.

14. The composition of Claim 13 wherein said *Escherichia coli* verotoxin is a recombinant toxin.

20 15. The composition of Claim 14 wherein said recombinant *Escherichia coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT1 sequence.

25 16. The composition of Claim 14 wherein said recombinant *Escherichia coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT2 sequence.

30 17. The composition of Claim 14 wherein said antitoxin is directed against a portion of at least one *Escherichia coli* verotoxin.

18. The composition of Claim 14 wherein said portion of *Escherichia coli* is selected from the group consisting of subunit A and subunit B of VT1.

19. The composition of Claim 14 wherein said portion of *Escherichia coli* is selected from the group consisting of subunit A and subunit B of VT2.

20. The composition of Claim 14 wherein said antitoxin is directed against a portion of at least one *Escherichia coli* verotoxin.

21. The composition of Claim 14 wherein said antitoxin is an avian antitoxin.

22. A method of treatment of enteric bacterial infections comprising:

10 a) providing:

i) an avian antitoxin directed against at least one verotoxin produced by *Escherichia coli* in an aqueous solution in therapeutic amount that is parenterally administrable, and

ii) at least one infected subject; and

15 b) parenterally administering said avian antitoxin to said subject.

23. The method of Claim 18 wherein said *Escherichia coli* is selected from the group consisting of *Escherichia coli* serotypes O157:H7; O1:NM; O2:H5; O2:H7; O4:NM; O4:H10; O5:NM; O5:H16; O6:H1; O18:NM; O18:H7; O25:NM; O26:NM; O26:H11; O26:H32; O38:H21; O39:H4; O45:H2; O50:H7; O55:H7; O55:H10; O82:H8; O84:H2; O91:NM; O91:H21; O103:H2; O111:NM; O111:H8; O111:H30; O111:H34; O113:H7; O113:H21; O114:H48; O115:H10; O117:H4; O118:H12; O118:H30; O121:NM; O121:H19; O125:NM; O125:H8; O126:NM; O126:H8; O128:NM; O128:H2; O128:H8; O128:H12; O128:H25; O145:NM; O125:H25; O146:H21; O153:H25; O157:NM; O163:H19; O165:NM; O165:19; and O165:H25

24. The method of Claim 22 wherein said antitoxin comprises antitoxin directed against at least one *Escherichia coli* verotoxin.

30 25. The method of Claim 22 wherein said antitoxin is cross-reactive with at least one *Escherichia coli* verotoxin.

26. The method of Claim 22 wherein said antitoxin is reactive against toxins produced by members of the genus *Shigella*.

27. The method of Claim 26, wherein said antitoxin is reactive against toxins
5 produced by *Shigella dysenteriae*.

28. A method for detecting *Escherichia coli* verotoxin in a sample comprising:

- a) providing:
 - i) a sample;
 - ii) an antitoxin raised against *Escherichia coli* verotoxin; and
 - iii) a reporter reagent capable of binding said antitoxin; and
- b) adding said antitoxin to said sample so that said antitoxin binds to the *Escherichia coli* verotoxin in said sample.

15 29. The method of Claim 28, wherein said antitoxin is an avian antitoxin.

30. The method of Claim 28, further comprising the steps of:

- c) washing said unbound antitoxin from said sample;
- d) adding said reporter reagent to said sample so that said reporter reagent binds to said bound antitoxin;
- e) washing said unbound reporter reagent from said sample; and
- f) detecting said reporter reagent bound to said antitoxin bound to the *Escherichia coli* verotoxin so that the verotoxin is detected.

25 31. The method of Claim 30 wherein said detecting is selected from the group consisting of enzyme immunoassay, radioimmunoassay, fluorescence immunoassay, flocculation, particle agglutination, and *in situ* chromogenic assay.

32. The method of Claim 30 wherein said sample is a biological sample.

30

33. The method of Claim 30 wherein said sample is an environmental sample.

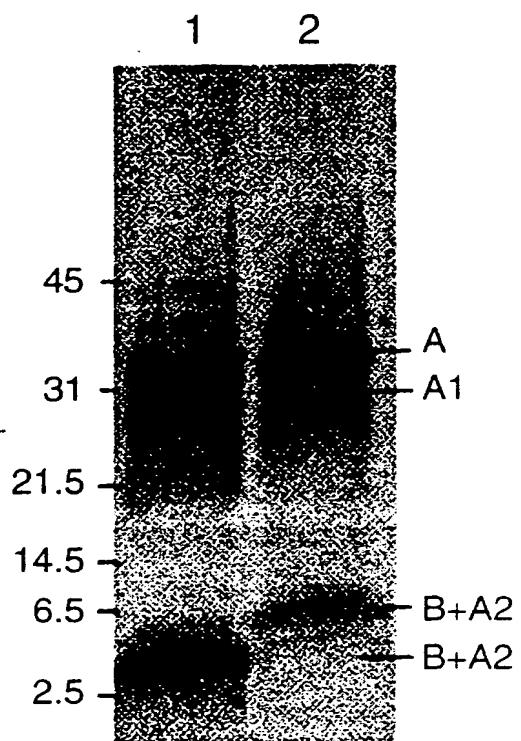


FIG. 1

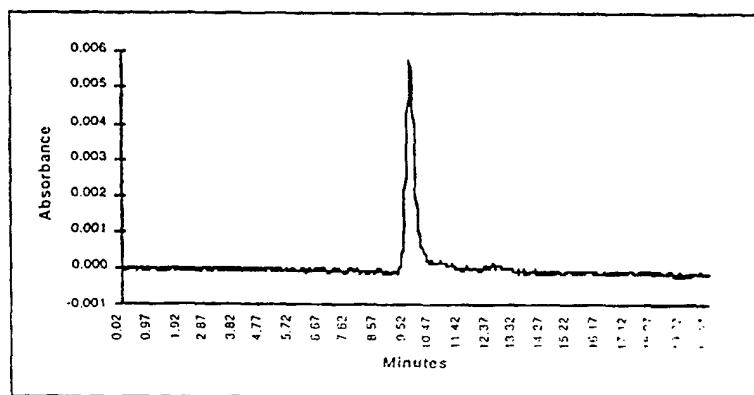
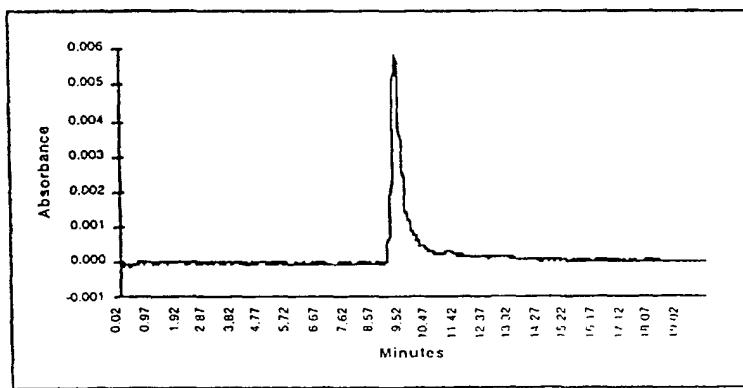
Figure 2.**HPLC of rVT1****HPLC of rVT2**

Figure 3.
rVT1 and rVT2 Toxicity in Vero Cell Culture

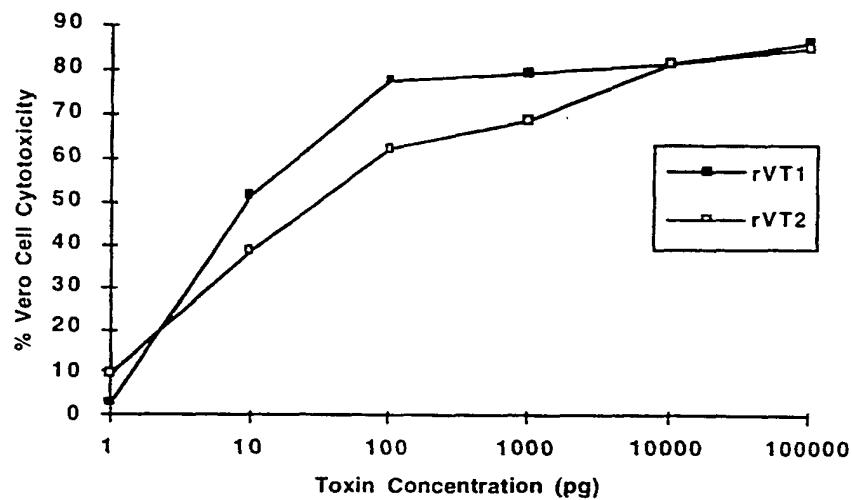


Figure 4.
EIA Reactivity of rVT1 and rVT2 Antibodies to rVT1

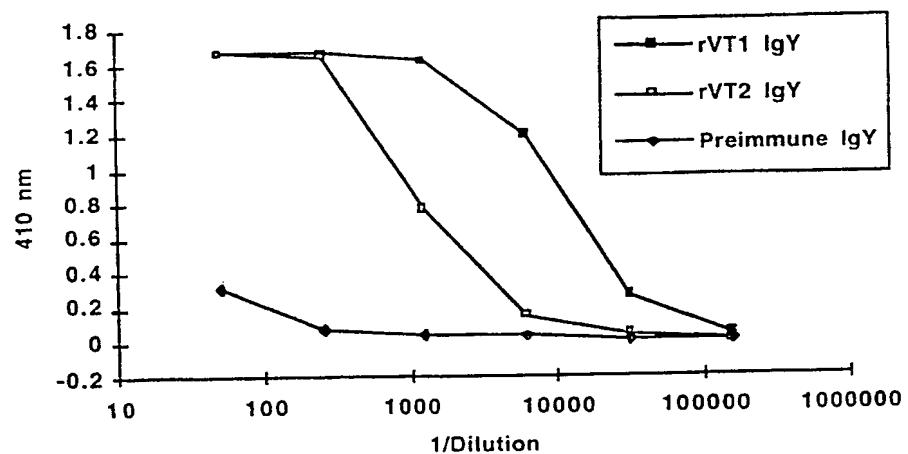
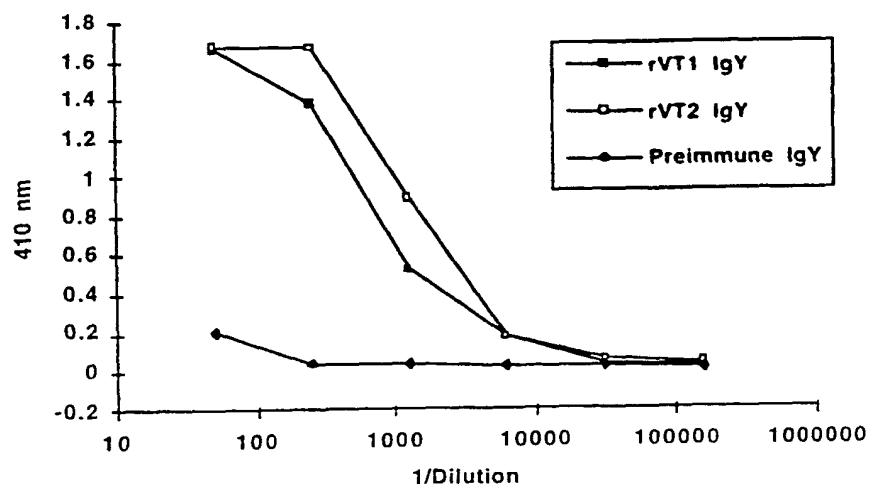


Figure 5.
EIA Reactivity of rVT1 and rVT2 Antibodies to rVT2



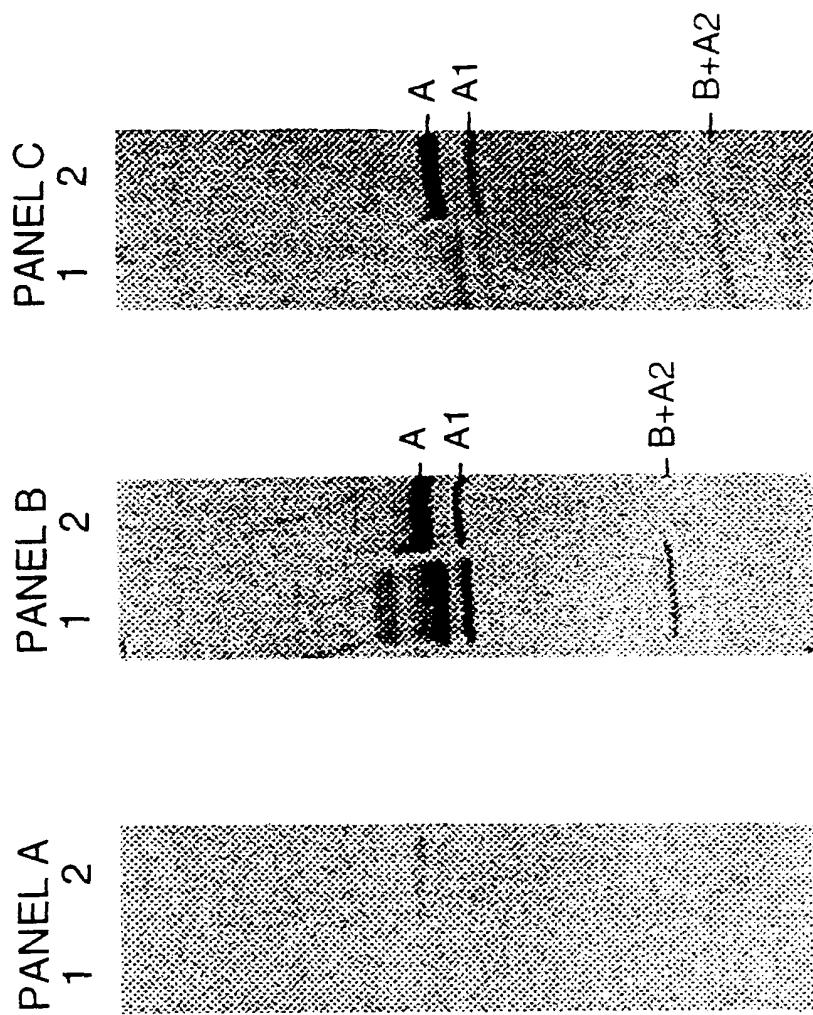


FIG. 6

Figure 7.
Neutralization of rVT1 Cytotoxicity in Vero Cells

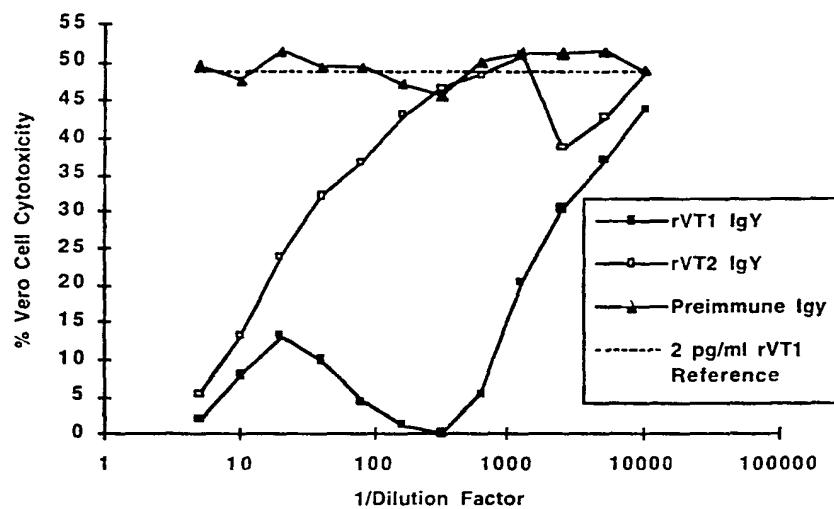
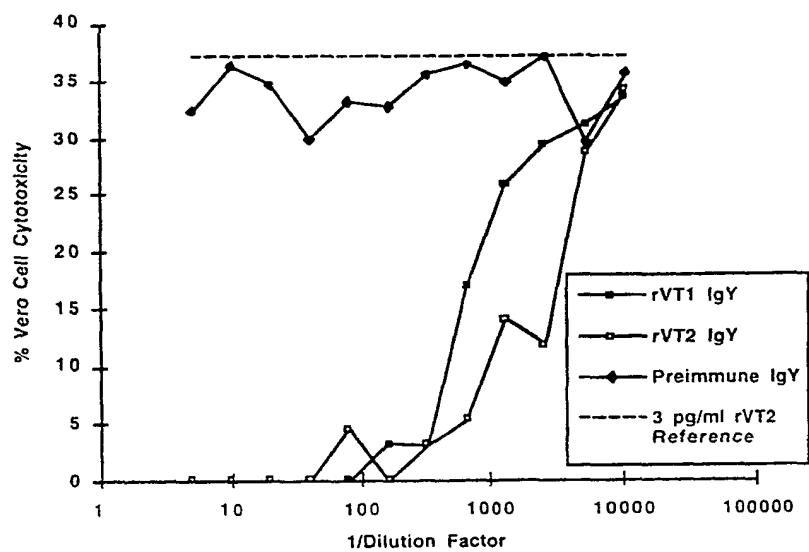


Figure 8.
Neutralization of rVT2 Cytotoxicity in Vero Cells



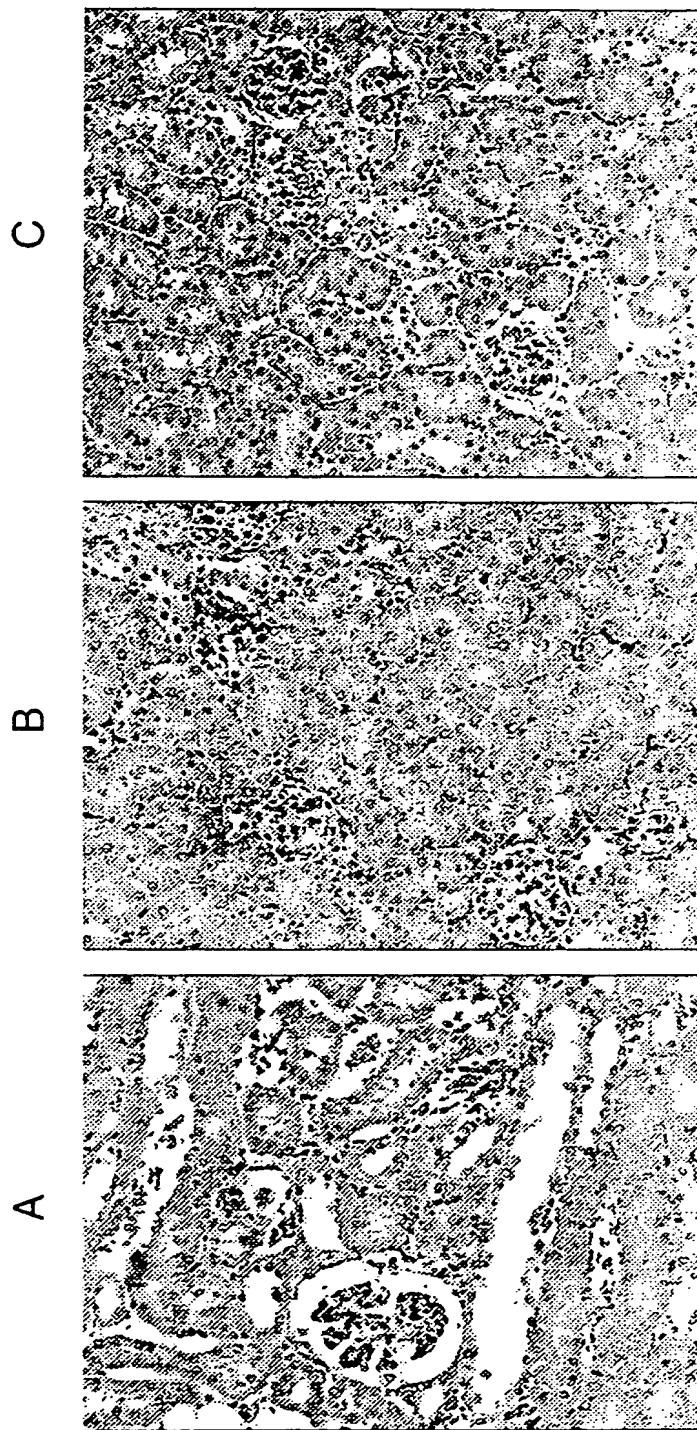
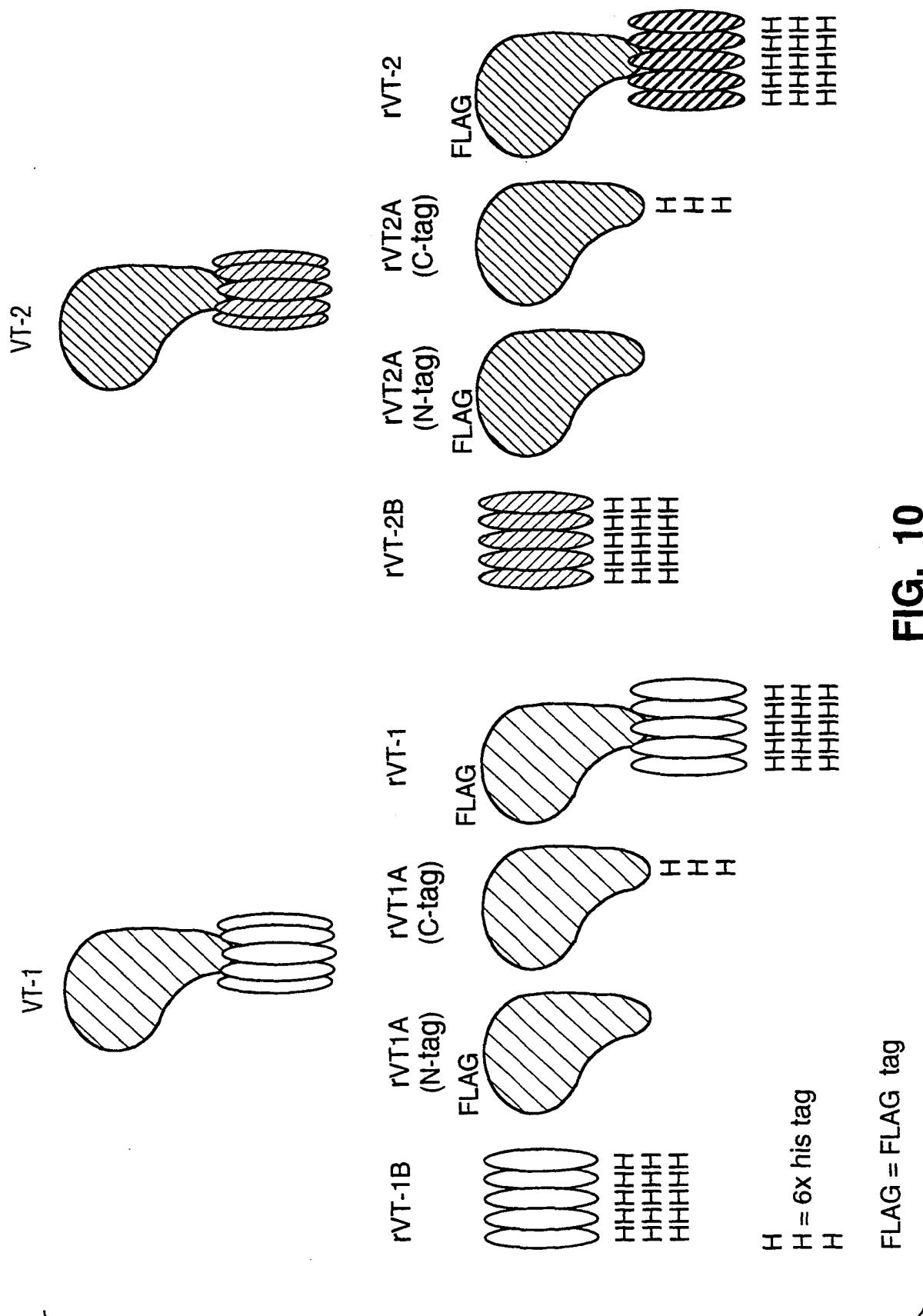


FIG. 9



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/04093

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 39/00, 39/02; G01N 35/537

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/134.1, 141.1, 150.1, 157.1, 164.1, 169.1, 192.1, 200.1, 236.1, 241.1, 801, 804, 809, 826; 435/7.37; 436/538, 542, 543-547

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BOYD et al. Serological Responses to the B Subunit of Shiga-Like Toxin 1 and Its Peptide Fragments Indicate that the B Subunit Is a Vaccine Candidate To Counter the Action of the Toxin. Infection and Immunity. March 1991, Vol. 59, No. 3, pages 750-757.	1-33
Y	US 5,326,559 A (MILLER) 05 July 1994, columns 4-7.	1-33
X ---	US 5,164,298 A (LINGWOOD et al) 17 November 1992, columns 10-13.	28, 30, 31, 32, 33 -----
Y	US 4,748,018 A (STOLLE et al) 31 May 1988, column 4, lines 25-55.	1-27 and 29 3, 21, 22, 29

Further documents are listed in the continuation of Box C. See patent family annex.

Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claims) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search	Date of mailing of the international search report
10 JULY 1996	27 AUG 1996

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCF Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer RACHEL FREED Telephone No. (703) 308-0196
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/04093

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,550,019 A (POLSON) 29 October 1985, column 4, lines 46-68.	3, 21, 22, 29
Y	US 5,204,097 A (ARNON et al) 20 April 1993, column 2, lines 1-16, column 3, lines 33-56 and column 5, lines 53-67.	2 and 14

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP93/04093

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/134.1, 141.1, 150.1, 157.1, 164.1, 169.1, 192.1, 200.1, 236.1, 241.1, 801, 804, 809, 826; 435/7.37; 436/538,
542, 543-547

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

BIOSIS, MEDLINE, APS

search terms: verotoxin, verocytotoxin, shiga, rvt1, rvt2, rslt1 or rslt2, vaccin? or treat?, recombinant

